The ANALYSIS *of* FOODS

ANDREW L. WINTON

Sometime State and Federal Chemist

AND

KATE BARBER WINTON

Sometime State and Federal Microscopist

With 208 Illustrations

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BOOKS ARE WEAPONS IN THE WAR OF IDEAS

THIS BOOK HAS BEEN MANUFACTURED IN ACCORDANCE WITH THE RECOMMENDATIONS OF THE WAR PRODUCTION BOARD IN THE INTEREST OF THE CONSERVATION OF PAPER AND OTHER IMPORTANT WAR MATERIALS.

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PREFACE

This book is built about the century-old methods for the determination of the constituents of foods in six groups. Some of the methods selected after thorough testing have been adopted by American national organizations or are standard in other countries, but others, although developed in laboratories of good repute and published in accredited journals, have yet to run the gauntlet. In the interest of those who are not professional food analysts, apparatus and reagents are briefly treated in the Introduction, illustrations of microscopic tissues are given at the beginnings of the chapters, and reaction equations are included in the descriptions of the methods. As practice examples for students, typical methods, listed after the Table of Contents, are described in explicit detail.

The analysis of methods has demanded quite as much attention as the analysis of foods. In fact the most difficult—in some cases almost hopeless—task of the compilers has been the separation of the method proper from its entanglement with experimental and discussion in journal articles and the piecing together of the parts to form a lucid and usable whole.

For brevity and convenience the terms test and method are arbitrarily applied to qualitative and quantitative methods respectively. A method is considered to be a procedure in which a basic reaction, a special reagent, or a physical operation is used for the first time for the particular purpose and a modification as a method that has been distinctly improved in accuracy or convenience without a change in the fundamental features; however, in the index, for brevity, this distinction is not made. The mere substitution of one solvent or oxidizing or reducing agent for another is not deemed worthy of special notice, but the adaptation of a method to a particular product is given due prominence. In the triple designation of a method, first the originator is given, second the distinctive reagent or reaction product, and third the class to which the method belongs. The word Process is used as a side head preceding the directions of a method; the word procedure is reserved for more general application.

Our gratitude for permission to reproduce cuts or for other courtesies is due Dr. W. W. Skinner, General Chairman, and Dr. Henry A. Lepper, Editor, of the *Journal of the Association of Official Agricultural Chemists* (also the "Methods of Analysis" of that Association), Dr. Walter J. Murphy, Editor of *Industrial and Engineering Chemistry* including *Analytical Edition*, the Board of Editors of the *Journal of Biological Chemistry*, Dr. W. F. Geddes, Editor of *Cereal Chemistry* (also the Committee of Revision responsible for "Cereal Laboratory Methods"), Dr. Louis Ehrenfeld, Editor of the "Methods of Analysis of the American Society of Brewing Chemists," and Dr. S. B. Wildrick, Editor of *The Chemist Analyst.*

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Company, E. H. Sargent & Co., Spencer Lens Company, Arthur H. Thomas Co., and Carl Zeiss, Inc., for electros or permission to reproduce cuts and tables.

Dr. D. D. Van Slyke not only allowed the use of the methods and cuts published in papers of which he is the senior author, but also graciously added new matter in conformity to more recent experience. "Physical and Chemical Methods of Sugar Analysis," third edition, by Dr. C. A. Browne and Dr. F. W. Zerban, was often consulted in preparing the manuscript, as were also *Chemical Abstracts* (Dr. E. J. Crane, Editor) and "The Merck Index," fifth edition (Dr. Joseph Resin, Editor).

Among the other food specialists who have generously aided us are Mr. H. S. Bailey of The Exchange Orange Products Company, Ontario, Calif. (fruit products); Mr. Raymond T. Bohn of the General Baking Company, N. Y. (flour); Dr. M. Fine of General Foods, Inc. (vitamins); Mr. Herbert C. Gore of Scarsdale, N. Y. (fruit products and coffee); Dr. B. G. Hartmann of Washington, D. C. (organic polybasic acids of fruits); Dr. Daniel Melnick of Food Research Laboratories, Inc. (vitamins); Dr. A. E. Paul of Chicago (colors); Dr. C. N. Frey and Dr. W. E. Stokes of Standard Brands, Inc. (leaven); and Mr. L. M. Tolman of Wilson and Co., Chicago (meat).

Professor Treat B. Johnson, a colleague of forty years since, also Professors Arthur J. Hill (chairman), Werner Bergmann, and Harold G. Cassidy, all of the Sterling Chemistry Laboratory of Yale University where much of our library work was done, have always been ready to answer difficult questions in their respective fields. Our daughter, Dr. Mary G. Winton, has given much attention to the organic nomenclature and other details.

The chance suggestion of Dr. Willis S. Hilpert of the Miner Laboratories, Chicago, shortly before the untimely end of a notable career, was the initial impetus that led to the undertaking. The memory of Professor Samuel W. Johnson, Liebig's honored student and the first American translator of Fresenius' "Qualitative and Quantitative Analysis," and Dr. Harvey W. Wiley, under both of whom we served, have been ever-shining beacons during the years of accumulating and classifying the material.

A. L. W
K. B. W

Wilton, Connecticut
November, 1944

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A SUGGESTED SHORT COURSE IN FOOD ANALYSIS

The methods selected are those most used and those featuring a wide variety of operations. By dividing a class into sections of six each, time may be saved in using the instruments and multiple apparatus. Each student should (1) prepare microscopic mounts (pp. 27–31) of scrapings and cross sections of natural vegetable foods and compare the tissues with those illustrated and (2) analyze a single product of each group by the following methods and compare the results with those in the tables and those obtained by other students on different products.

Cereal Foods. Grind samples to pass a 1-mm. sieve (p. 2). Employ the methods described in Part I, C2, for water, fat, fiber, and ash. Determine nitrogen by the Kjeldahl-Wilfarth method (Part I, C1c) and calculate the protein by the factor 6.25. Obtain nifext by difference.

Oils and Fats. In representative samples (including fat separated from melted butter) determine specific gravity (p. 486), refractive index (p. 487), melting point if a fat (p. 485), saponification number (p. 489), Hanuš iodine number (p. 498), Reichert-Meissl number (p. 491), and Polenske number (p. 493). Make qualitative tests for cottonseed oil by the Halphen method (p. 529) and for sesame oil by the Baudouin method (p. 535).

Fruit Products. Determine solids (p. 573), sucrose (pp. 609–612), and acidity (p. 565) in fruit juices and jellies. Determine ascorbic acid in fruit juice by titration with iodine solution (p. 589) and by the Tillmans method (p. 367). Add cochineal solution to a fruit juice and apply the Robin test (p. 399). Make wool-dyeing tests by the Arata method (p. 401) on portions of a fruit juice colored with various natural and coal-tar dyes. Make spot tests on the dyed fabric (pp. 406–408).

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Milk. Determine specific gravity by the Quevenne lactometer (p. 711), solids by the dish method (p. 714), solids and fat by the asbestos gravimetric method (pp. 715, 726), and fat by the Babcock centrifugal method (p. 729). Calculate the solids-not-fat by the Babcock formula method (pp. 716, 717). Determine protein ($N \times 6.33$) and ash (p. 66) and calculate lactose by subtracting from the solids the sum of the fat, protein, and ash. Apply the Hehner (p. 758) and the turmeric (p. 759) tests to milk containing 40% formaldehyde (0.2 ml. per liter) and borax (1 g. per liter) respectively.

Butter. Determine water, fat, curd, and salt (pp. 775, 776).

Meat. Mix 1000 g. of ground Hamburg steak with 2 g. of sodium sulfite and determine sulfur dioxide by the gravimetric method (p. 413).

Flavoring Extracts. In lemon extract determine volatile (essential) oil by the Mitchell

method (p. 909) and citral by the Hiltner method (p. 910). In vanilla and imitation vanilla extracts determine vanillin and coumarin by the Hess and Prescott method (p. 916), and lead number by the Winton and Lott method (p. 921), also vanillin by the Folin and Denis method (p. 918).

Baking Powder. Determine total carbon dioxide by the Heidenhain method (p. 932). Make qualitative tests for constituents.

INTRODUCTION

EQUIPMENT

APPARATUS

In this section only brief mention is made of numerous optical instruments of recent introduction, adequately described and illustrated in manufacturers' pamphlets.

Sampling and Grinding Apparatus

Too often improper sampling, grinding, or mixing nullifies an accurate analysis. This is particularly true of natural products or artificial mixtures containing parts of radically, different composition such as fibrous hulls mixed with starchy endosperm, or watery and saccharine pulp mixed with solid starchy or fatty seed tissues.

Sampling Apparatus. Material in bags is conveniently sampled by a brass tube 2 to 3 ft. long with a slot extending from near the cross-piece serving as a handle to the conical tip. When introduced into the bag diagonally, with the slot underneath, then turned halfway around, the tube fills with a representative fraction of the whole content.

A good rule is to sample in the above manner one bag in ten, a hundred, or more, depending on circumstances, then mix well and take a subsample by removing portions here and there with a flat scoop having parallel sides. Further subsamples may then be taken with continued precautions. The utmost care must be used to avoid gain or loss of moisture.

A number of machines for automatically dividing into several parts various granular substances, including seeds and meal, are supplied by apparatus houses. The material poured into a hopper enters several spouts, either directly or after impinging on a cone.

Iron Mortar. For certain materials, particularly portions of a sample separated by sifting, an iron mortar is more useful than a porcelain one. A sheet metal cover with a hole for the pestle prevents loss of flying particles of horny material (Fig. 1).

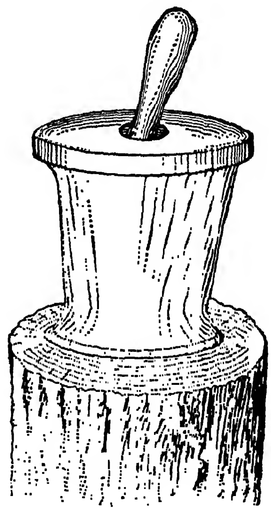


Fig. 1. Iron Mortar with Pestle and Sheet Iron Cover.

Drug and Coffee Mills. These are supplied for both hand and electric power. They are useful for samples of considerable size.

Wiley Mill.[1] * A need that the writers for many years experienced is now well filled by the Wiley mill. It reduces, by a shearing action, to a finely comminuted condition and at the same time sifts a variety of dry and fibrous materials without appreciable heating or exposure to the outside atmosphere, thus avoiding change in moisture content. It is supplied by Arthur H. Thomas Company, Philadelphia, in three models (Standard, Intermediate, and F.R.I. Micro). The Intermediate model is provided with a motor, sieve top delivery tubes of 20, 40, and 60 mesh, and glass receiver (Fig. 2).

Sieves. A set of sieves with round holes 0.5, 1.0, and 2.0 mm. in diameter is often of service. As a rule samples ground to pass a 1-mm. mesh are of suitable fineness for analysis, although starchy samples require finer grinding. Portions of a sample of different fineness and physical character may be separately comminuted and then mixed. (Fig. 3.)

Food Chopper. The chopper (Fig. 4) made for family or butcher use is often in demand in the laboratory for grinding both meat and vegetable materials. It serves alike for dry or wet, hard or soft substances.

Sample Bottles. Dry and wet samples are conveniently stored, if large, in fruit jars and dry samples, if small, in wide-mouth, glass-stoppered, 4-ounce bottles.

Steam Baths

In a small laboratory, a water bath, such as an enameled soup bowl, provided with a set of rings and heated by a Bunsen burner, is suitable for evaporation. A battery of such baths, connected with a tank kept at a constant level by a ball cock, or better still with a steam supply, is regarded as a necessity for work on a considerable scale. As used in this volume, the terms steam bath and boiling water bath are synonymous, the former being preferable because of brevity.

In conducting certain processes, a flask, beaker, or retort is heated by submerging in water at various temperatures, including boiling. The term water bath applies in this instance.

Drying Apparatus

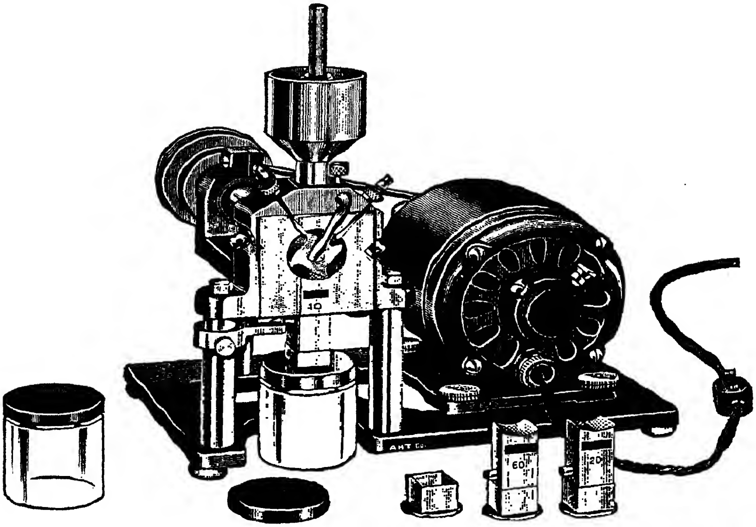
Water Ovens. The familiar double-walled water oven is so frequently in demand that connection with a constant level attachment is highly desirable. The same attachment may also supply water to a battery of water baths at the proper level, if live steam is not available. Under the most favorable conditions, the temperature of the interior falls short of 100°, hence "drying in a boiling water oven" is a more guarded statement than "drying at the temperature of boiling water."

A steam oven over the water oven serves the double purpose of a condenser and a drying oven at a temperature somewhat lower than that of the lower oven.

Constant Temperature Air Ovens. The Freas Oven (Eimer and Amend, New York; E. H. Sargent and Co., Chicago), the Dekhotinsky Oven (Central Scientific Company, Chicago), and the Weber Oven (Arthur H. Thomas Co., Philadelphia), as well as other makes, heated by gas or electricity, are so regulated as to keep the drying compartment at any desired temperature within narrow limits between 35 and 150° or even higher. The absence of the moist air due to the evaporation from a water oven also contributes to thorough drying.

Constant Temperature Vacuum Ovens. Drying *in vacuo* at 100° or lower is often desirable as a means of avoiding oxidation of fats, decomposition of levulose which takes place at temperatures above 70°, or other changes, as well as securing a more complete desiccation. In addition to ovens arranged

* Superior figures refer to "References" at the end of the section. See p. 17.



Courtesy of Arthur H. Thomas Company, Philadelphia

Fig. 2. Wiley Intermediate Laboratory Mill and Motor. Center, mill with 40-mesh sieve-tube, adapter, and glass receiver. Left, glass receiver. Right, metal receiver and 60- and 20-mesh sieve-tubes.

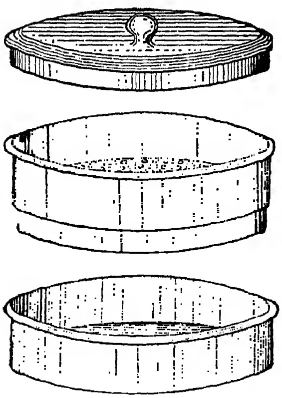


Fig. 3. Sieve with Cover and Receiver.

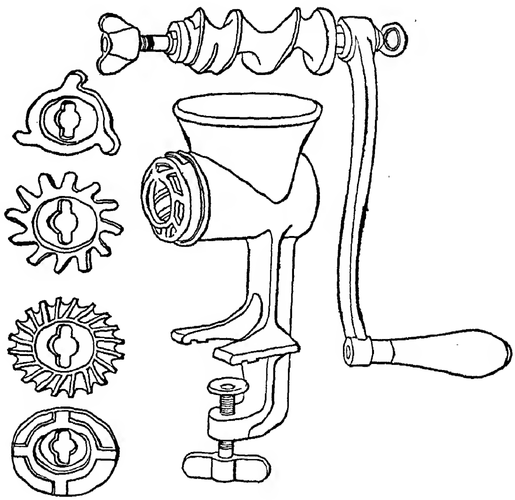


Fig. 4. Universal Food Chopper.

for vacuum drying only, constant temperature ovens of the Freas, Dekhotinsky, and Weber types with a removable compartment are supplied. All vacuum ovens require for their efficient operation a mechanical air pump which exhausts more completely than a water filter pump.

Vacuum Desiccators. Water determination in many materials containing volatile constituents cannot be carried out with the aid of heat. For such materials the vacuum desiccator has extensive application. It consists essentially of an ordinary desiccator with a tubulature connected with a water pump or, better, with mechanical vacuum apparatus. An efficient drying tube or jar is inserted between the desiccator and the pump.

Furnaces and Heaters

Muffle Furnaces. For laboratories not supplied with illuminating gas, an *electric muffle furnace* is practically indispensable. It not only is efficient for reducing organic materials to a white or gray ash, but also furnishes a smokeless heat for igniting precipitates. A gas muffle furnace is recommended only for the rare situations where an electric current is not available.

Combustion Furnaces. A gas or electric furnace designed primarily for carbon and hydrogen combustions serves also for Dumas nitrogen combustion if supplemented by an azotometer.

Multiple Heaters. A multiple stand for Bunsen burners or electric heaters, such as is provided for Kjeldahl digestions, is equally serviceable for other operations depending on easily regulated heat.

Stands and Supports

Supports for filters, burets, test tubes, condensers, etc., need only be mentioned.

Separatory Funnel Stands. Split rings for iron supports are suitable for single extractions, but the multiple adjustable stand shown in Fig. 203, devised by one of us, is desirable for a series of analyses. It is arranged for filtering into each separatory funnel and drawing from one into another. Each one is introduced at the constriction just above the cock, after which it drops into the beveled hole where it is held firmly.

Centrifuges

Laboratory centrifuges serve for the determination of fat in milk and essential oil in extracts, also extensively for separating a clear supernatant liquid from a precipitate in a beaker, tube, or bottle or an emulsion in a separatory funnel. The International Centrifuge (International Equipment Company, Boston) is provided with heads and carriers for the various analytical operations. Certain modern operations depend on centrifuging rather than filtration for their success. The centrifuges made for the dairy laboratory (Figs. 162 and 163) usually are not provided with carriers for more general use.

Metal Ware

Laboratory utensils of platinum, nickel, and aluminum, so far as possible, should replace those of porcelain or glass. *Small flat-bottom platinum dishes* are suitable for incinerating dry powders for ash determinations; the square form (about 4 cm. in diameter) is economical of space in a muffle furnace. *Large round platinum dishes* are used for reducing to ash considerable portions of food samples for ash analysis, as well as other purposes. *Platinum crucibles*, particularly those of Gooch type (covers for most purposes unnecessary), in the long run are more economical than porcelain in a large laboratory; if they become damaged, they may be dissolved for the preparation of platinic chloride solution. Quartz ware is a satisfactory substitute for many purposes. *Flat-bottom round*

aluminum dishes (5 to 6 cm. in diameter) are of wide usefulness, as in moisture determination; if used with a slip-in cover, the desiccator may often be dispensed with.

Glass Ware

Passing over beakers, flasks, crystallizing dishes, funnels, and other common glass utensils, the *pear-shaped separatory funnel* (Fig. 203) is a highly important piece of apparatus in the food laboratory, the 125-, 250-, and 500-ml. sizes being in constant demand. Globular and cylindrical forms are less convenient.

Burets suitable for nitrogen determination are pictured in Fig. 35. For general use the form attached to the 5-pint green acid bottle is convenient. *Single-mark pipets* for 1 to 100 ml., also *Mohr pipets* graduated to subdivisions of a milliliter are indispensable in aliquoting and measuring reagents. For microanalyses, both burets and pipets of narrow bore, graduated to 0.01 ml. or less, are available. *Graduated cylinders* of 10, 100, and 500 ml. and *volumetric flasks* of 50, 100, 200, 250, 500, and 1000 ml. meet the requirements of methods described in this book.

Sintered glass Gooch crucibles, porosity E, are supplied by Ace Glass Incorporated, Vineland, N. J., and recommended for filtering barium sulfate and other finely divided precipitates. Filter tubes of porosity B or C may be used in the Johnson fat extractor.

Extraction Apparatus

Continuous Extractors for Powders. The *Johnson extractor* appears in Figs. 40, 41, and 159.

The *Knorr extractor* is for use with a special flask provided with a channel for a mercury joint.

The *Soxhlet extractor*, although much used and well suited for the extraction of large amounts of the substance (10 g. or more), requires a large amount of the solvent as compared with the few milliliters sufficient for the two foregoing extractors. The substance is contained in a filter-paper cartridge folded over the end of a flat-bottom vial or in a filter thimble. Each time the extraction chamber is filled, the solvent siphons over into the extraction flask.

Rubber Committee Extractor.[2] The advantage of this form is the non-breakable metal coil condenser which is inserted within the wide-mouth extraction flask. A vapor-tight connection is secured by ground surfaces.

The *Mann extractor* (Fig. 42), although lacking some of the desirable features of other extractors, is suited for cold extraction since the solvent in hot gaseous form does not come in contact with the material.

Continuous Extractors for Liquids. *Palkin Extractors.*[3] Simple and all-glass forms for heavy and light solvents are described. In the simple forms the condensed solvent bubbles through the solution in liquid form; in the all-glass forms it enters the liquid wholly or in part in vapor form (Figs. 169 and 170).

Chromatographic Apparatus

Interjoint chromatographic tubes for adsorption methods, designed by Zechmeister and Cholnoky,[4] are supplied by the Scientific Glass Apparatus Co. of Bloomfield, N. J., either with perforated glass disks or with fritted glass disks sealed to the glass above the joint.

Suitable adsorbants are packed in the tube and a solution of the sample is sucked through the column, thus permitting adsorption according to their respective affinities. The separation is rendered more effective by finally sucking a solvent through the column. When pressed out of the tube, the individual color zones may be separated with a knife and the colors eluted with suitable solvents. If the adsorbed substances are colorless, they

are rendered evident by treatment with chromogenic reagents or examination in ultra-violet light.

Densimeters

Hydrometers. Wide range hydrometers, covering all densities either above or below that of water, are useful in the preparation of reagent solutions or the approximate measurement of the specific gravity of samples. Sets of accurately calibrated hydrometers with narrow range are better suited for general analytical work. Special hydrometers, designed for the milk, sugar, alcohol, and vinegar industries and for government inspection and gauging, have wide application in controlling production, detecting adulteration, and establishing bases for taxation.

The *Quevenne lactometer* (Fig. 156, *B*), which for convenience drops 1.0 from the specific gravity and moves the decimal point two places to the right, and the *Brix scale*, which automatically converts specific gravity into percentages of sucrose, are desirable simplifications, but the arbitrary *Baumé scale*, like the English system of weights and measures, has little to commend it except long usage.

A thermometer, forming an integral part of the hydrometer, although adding to the expense and fragility, is often a desirable convenience.

Pycnometers. The *Sprengel U-tube type* is perhaps the most accurate.

Bottle pycnometers are obtainable in sizes from 10- to 100- ml. capacity with either (1) capillary stoppers, both bottle and stopper being filled completely at the graduation temperature, or (2) a graduated neck with either a single mark for filling at a definite temperature or a series of marks for filling at different temperatures.

For alcohol determination, the writers prefer a 100-ml. bottle pycnometer with a flat bottom and a neck of about 5 mm. inside diameter (Fig. 144). Such a pycnometer resembles a Babcock milk-test bottle, but has a shorter neck. A narrow adapter tube permits distillation directly into the flask which, after cooling in water of the standard temperature, is filled to the mark and weighed.

Viscometers

The *MacMichael Viscometer* is a standard precision instrument. The *Gibson and Jacobs Viscometer*, as modified by Oppen and Schuette (Fig. 142), may be constructed in the laboratory.

Balances

In addition to the usual chemical balance, which serves also for many semi-micro methods, a special balance is usually necessary if micro gravimetric methods are undertaken. Many, however, of the micro methods are volumetric or colorimetric which are carried out with narrow-bore burets or colorimetric apparatus.

Pan Scales, weighing up to 1 kg. and accurate to a fraction of a gram, are useful in weighing a large charge of coarse material, as well as in making up reagents.

Westphal Balance. The specific gravity of oils, melted fats, and sirups is conveniently and accurately determined by the Westphal balance with the added advantage that the plummet may be lowered into the sample bottle or into a cylinder that is more easily cleaned than a pycnometer. By using a jacketed cylinder, the desired temperature may be maintained.

The plummet is suspended from the end of the long balance arm and the needle is adjusted to the zero point by a screw counterweight. Notches on the arm are numbered up to 10, each notch representing decimals of specific gravities in the first, second, third, or fourth places, depending on which of the pince-nez-shaped weights is hung thereon either directly or on the hook of another

weight. Thus, the heaviest weights on notches 5 and 4, plus the next smaller on notches 5 and 3, plus the next on notches 7 and 1, and plus the last on notches 3 and 1 equal specific gravity 0.9884 at the temperature shown by the thermometer immersed in the liquid.

Oils and fats are removed from the plummet by naphtha and sirups by warm water.

Hydrogen Ion Electrometers

The hydrogen electrode or its equivalent, in conjunction with the calomel electrode, has come into general use for the determination of hydrogen ion concentration. Definite results are read on a dial either as millivolts or pH equivalents, whereas in tests by indicators the color change is interpreted in terms of pH range within more or less narrow limits. Either method, as a rule, yields figures nearer the truth than those obtained by titration. Portable assemblies with glass and calomel electrodes, together with explicit instructions, are now made by Leeds and Northrup, Philadelphia, Pa., Coleman Electric Company, Maywood, Ill., and other American manufacturers.

The *hydrogen electrode* usually is made of platinum coated with platinum black, which, in a solution charged with hydrogen, absorbs the gas to such an extent that the action in the electric current is practically that of hydrogen alone.

The *quinhydrone electrode*, although a convenient substitute for the hydrogen electrode, has its limitations. It is, however, of value when oxidizing or reducing substances and certain other sources of error are absent. An uncoated platinum electrode is used, the quinhydrone being added to the solution until saturated. On hydrolysis, quinone and hydroquinone are formed and the equilibrium potential, representing the ratio of these two products, is determined and the pH is obtained by calculation.

The *glass electrode*, sent out ready for use, now takes the place of the hydrogen electrode which, however, is still the recognized standard. The construction and contents of the glass electrode differ with the type of the assembly and the manufacturer, but it is recognized that all glass electrodes undergo irregular changes, known as assembly potential (A.P.), necessitating frequent correction by comparison with standard apparatus. A reference electrode is sent out with the assembly.

The *calomel electrode* in its simplest form is a test tube with a sealed-in platinum wire passing through the bottom to the electric connection. By a narrow tube passing through one hole of a double-bored rubber stopper, the contents of the tube are connected with the solution of the sample in a beaker, and by a tube passing through the other hole with a bottle on a shelf. At the bottom of the tube are placed a few milliliters of metallic mercury, then a layer of a pasty mixture of calomel (mercurous chloride) and a little normal potassium chloride. Normal potassium chloride, saturated with calomel, is poured into the bottle on the shelf and allowed to flow into the calomel tube until it is practically filled; then the flow is halted by a stopcock. Each time the calomel tube is used, a little of the potassium chloride solution is allowed to run into it and thus force some of the solution out through the other tube. As now prepared, the calomel tube is filled at the factory, but needs the addition of extra potassium chloride from time to time.

Redox electrodes are also provided for the determination of oxidation-reduction potential.

Refractometers

Abbé Refractometer. Of the physical values determined in fats (notably butter and lard), fatty oils, and essential oils, the refractive index, as determined by the Abbé

refractometer, ranks with the specific gravity in importance.

Special forms are used for the determination of fat in milk, various meals, and powders, after solution of the fat in an organic solvent, as well as of solids in molasses, sirup, and other saccharine liquids.

Originally supplied only by Zeiss, high grade instruments are now made by the Bausch & Lomb Optical Co., Rochester, N. Y., and the Spencer Lens Company, Buffalo, N. Y. (Figs. 5 and 6).

Dipping (Immersion) Refractometer. This type (Fig. 7) serves for the examination of non-fatty, non-viscous liquids such as milk serum in the detection of watering of the milk or alcoholic liquors suspected of containing methanol (wood alcohol). Bausch & Lomb and Spencer instruments now rank with the original form made by Zeiss.

Calculation of results, obtained with both the Abbé and the dipping refractometer, is facilitated by tables supplied by the manufacturers or given in reference works.

Saccharimeters

A discussion of the theories of polarization of light and a description of a simple type of polariscope appear in all works on physics. The various types of instruments adapted for the determination of sugars are described in detail by Browne and Zerban.[5] Because of the war, the importation of the well-known European instruments made by Schmidt and Haensch and by Frič has been interrupted; the Bausch & Lomb saccharimeter, however, is available (Fig. 137).

Colorimetric and Spectrographic Instruments

Methods involving measurement of colors and ultra-visible rays are of particular importance in the determination of vitamins, minor mineral constituents, and traces of toxic substances. Comparison tubes and simple colorimeters have long been used, but precision instruments, utilizing monochromatic light of selected wave lengths of maximum absorption for colored solutions obtained in characteristic reactions, or absorption curves of ultra-violet rays, have recently been perfected by American manufacturers cooperating with leading physicists.

Colorimeters in the broad sense include all instruments for measuring colors, but in the strict sense are restricted to those measuring the true color of liquids, that is, the transmission of white light.

Photometers are colorimeters employing light approximately monochromatized by glass light filters which should not be confused with the standard matching slides or disks. They are either visual or photoelectric.

Fluorometers are photometers for measuring fluorescence.

Spectrophotometers are visual or photoelectric photometers in which the light is quite accurately monochromatized by prisms or gratings.

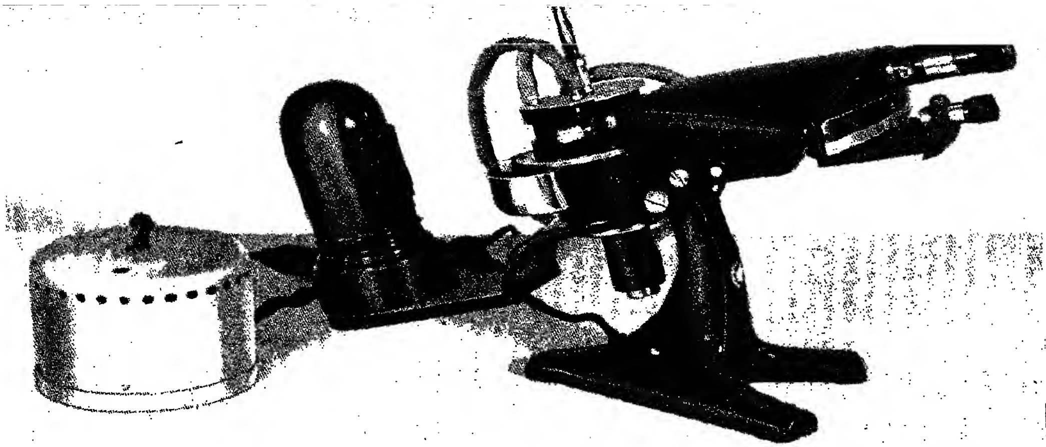
Spectrographs. Since ultra-violet spectra of vitamins A and D are devoid of color, they are photographed together with standard spectra in special instruments known as spectrographs.

Classification. The instruments here considered may be classified as follows:

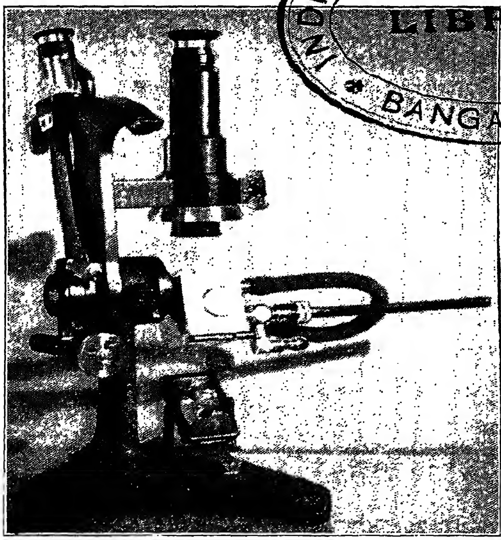
A. Colorimeters

I. Liquid standards.
   (1) Matching by removal of a portion of the liquid.
   (2) Matching by raising or lowering of a plunger.
   (3) Matching by changing the width of the light slit.
   (4) Matching by changing the light distance.

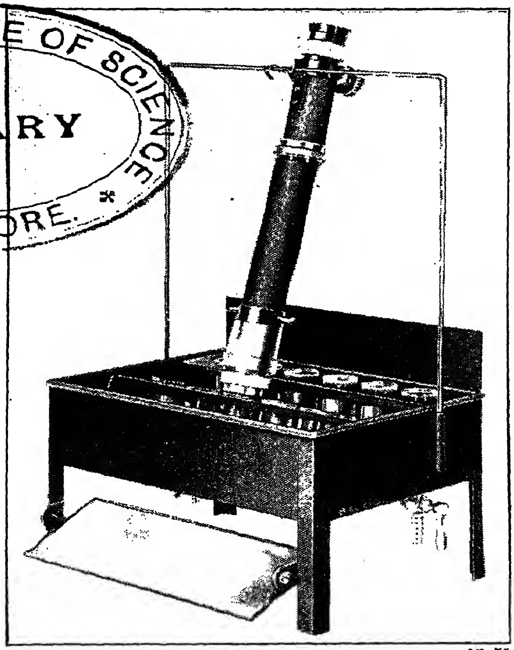
II. Glass standards.
   (1) Comparison with color slides.
   (2) Comparison with color disks.



Courtesy of Bausch & Lomb Optical Co., Rochester, N. Y.
FIG. 5. Bausch & Lomb Precision Laboratory Refractometer.



Courtesy of Spencer Lens Company, Buffalo, N. Y.
FIG. 6. Spencer Standard Abbé-Type Refractometer.



Courtesy of Bausch & Lomb Optical Co., Rochester, N. Y.
FIG. 7. Bausch & Lomb Dipping Refractometer and Constant Temperature Bath.

B. Photometers (Monochromatism by Color Filters)

I. Visual comparison.
II. Photoelectric measurement.

C. Fluorometers

D.

I. Monochromatism by prisms.
II. Monochromatism by gratings.

E. Spectrographs

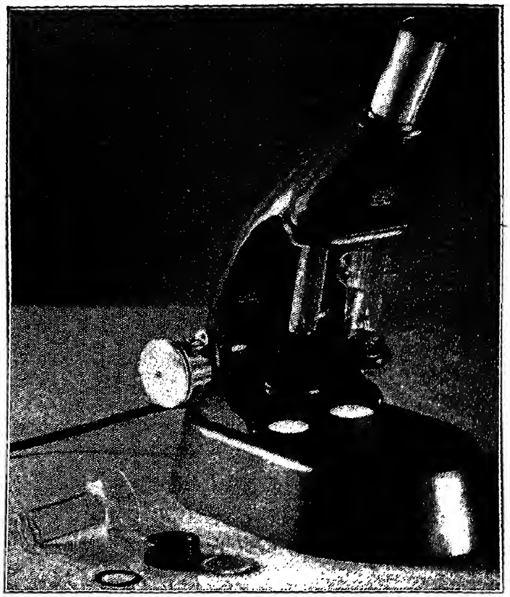
I. Dispersion by quartz prisms.
II. Dispersion by gratings.

Colorimeters

Visual colorimeters are subdivided into those comparing the unknown solution with (1) the known solution directly (liquid standard) and with (2) colored slides or disks which may be standardized against the known solution. The colorimeter reading takes the place of comparison of the unknown with a series of knowns in uniform Nessler tubes, as long practiced in water analysis.

Colorimeters for Liquid Standards (Figs. 8 to 10). Comparison is usually made in companion tubes, one for the known, that is, the standard solution containing in a conventional volume a definite amount of the substance being determined together with the color-forming reagent, the other for the unknown, that is, the extract or solution of a weighed amount of the sample, or aliquot, treated with the same amount of the color-forming reagent as the known and made up to the same volume. Matching the colors is carried out (1) by removing a portion of the liquid from one of two Nessler (color comparison) tubes by a pipet, or from one of two Hehner tubes by the cock near the bottom, (2) by raising or lowering plungers in the tubes (e.g., Duboscq, Schreiner, Spencer, and Klett colorimeters), (3) by varying the size of the light apertures (e.g., Pulfrich photometer used as a colorimeter), or (4) by varying the distance of the illuminating lamp (e.g., certain European instruments).

In the Nessler and Hehner tubes and the Schreiner colorimeter the two liquids are seen as circles, in the Duboscq, Spencer, Klett, and Pulfrich (Zeiss) colorimeters as halves of a circle, thus facilitating more accurate matching.



Courtesy of Spencer Lens Company, Buffalo, N. Y.

Fig. 8. Spencer Colorimeter.

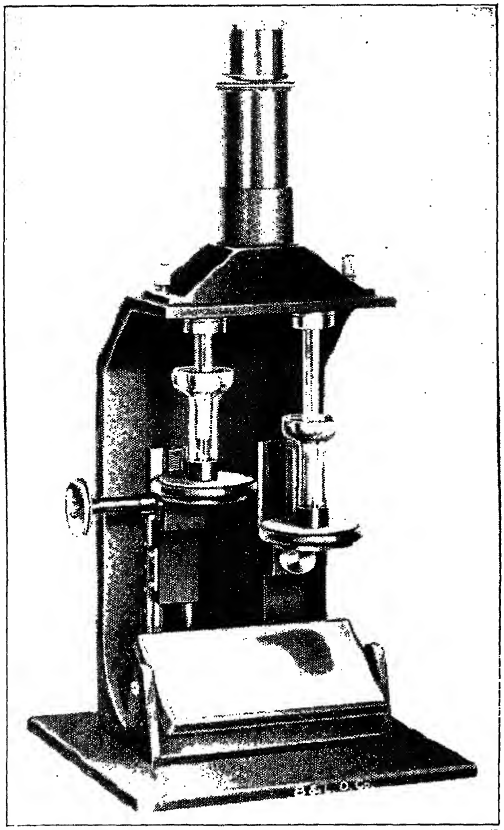
If the solution obeys Beer's law, calculation from the height of the liquid column in liquid removal or plunger instruments is by rule of three, thus:

mg. in unknown : mg. in known :: mm. of known : mm. of unknown

hence,

$$\text{mg. in unknown} = \frac{\text{mg. in known} \times \text{mm. of known}}{\text{mm. of unknown}}$$

The *Pulfrich photometer* (Fig. 10), unlike other visual instruments described, depends for matching the intensity of color or fluorescence on varying the size of the diaphragm



Courtesy of Bausch & Lomb Optical Co., Rochester, N. Y.
FIG. 9. Bausch & Lomb Duboscq-Type Micro Colorimeter.

aperture. A diaphragm photometer has higher light-gathering power than instruments employing polarization for light reduction; also it is obviously shorter than those depending on varying the distance of the light source. The relation of transmission read on the black scale to extinction (α_E) read on the red scale is represented by the following equation:

$$\alpha_E = 2 - \log \alpha_D$$

In colorimetric and absorption measurements, the extinction represents the degree of light absorbed, and if the solution obeys Beer's law, as is usual, extinction is directly proportional to the vertical or transverse thickness of the liquid and the concentration.

Colorimeters with Colored Glass Standards. These employ either (1) rectangular slides (e.g., Lovibond tintometer) or (2) disks (e.g., Hellige color comparator).

By standardizing the slides or disks against solutions of the known, the latter may be dispensed with in subsequent determinations.

The *Lovibond tintometer* (Fig. 205), designed primarily as an instrument for determining the color values of liquid and solid products, particularly as an aid in maintaining uniformity, may be used indirectly in colorimetric analysis. Following the laws of chromatics, the slides may be combined so as to match any possible color, each with a range from light to dark. For example, equal values of red and yellow produce orange; of red and blue, violet; of blue and yellow, green; and of red, yellow, and blue, neutral tint (gray to black). When they are combined in unequal values, various shades are produced. The following equations illustrate a few combinations, the depth and darkness of color being directly proportional to the values of the mixtures:

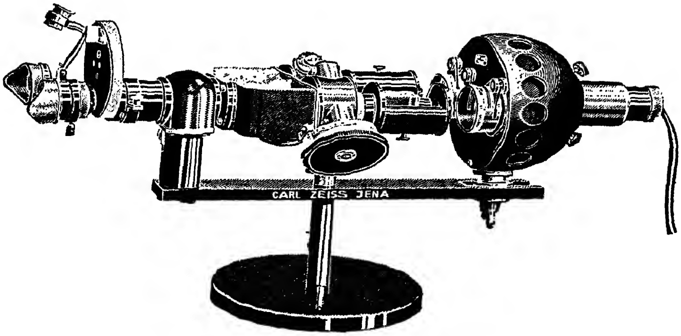
$$5.0R + 5.0Y = 5.0O$$

$$0.8R + 1.8Y = 0.8O + 1.0Y$$

$$3.0R + 3.0Y + 3.0B = 3.0N$$

$$0.8R + 1.8Y + 5.0B = 0.8N + 1.0G + 3.2B$$

$$10.0R + 8.0Y + 9.0B = 8.0N \quad 1.0R$$



Courtesy of Carl Zeiss, Inc., New York

G. 10. Pulfrich Photometer.

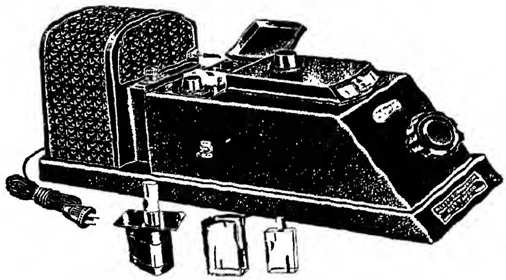
in which R is red, Y is yellow, B is blue, O is orange, G is green, V is violet, and N is gray (neutral tint).

Note. The following hints are for students unfamiliar with colorimetric measurements: Choose a soft but sufficient light, best at a north window or reflected from the north sky; never use direct sunlight. Practice at first with the same solution in both tubes. Do not use too high columns, as it is difficult to match deep colors. Avoid straining the eyes; adjust the tubes rapidly until the colors match approximately, then look away and when the eyes have rested a moment make the final adjustment in about 5 seconds. Do not attempt colorimetric work when the light is poor, when your eyes are tired, or when you are hurried or otherwise mentally disturbed.

Hellige Comparator (Hellige, Inc., Long Island City, N. Y.). Interchangeable disks of Textolite (a corrosion-resistant molded plastic manufactured by the General Electric Company), each with 9 or 10 standard glass color disks in steps arranged about the center, are supplied for the analysis and pH control of water. Separate disks are provided for the determination of nitrogen, in the form of ammonia, nitrite, and nitrate, chlorine, iron, oxygen, manganese, lead, phosphate, silica, and sulfides by suitable steps; also for 22 pH indicators with color standards by 0.2 steps. Disks for a wide range indicator (pH 1 to 11) are also provided. Series for both daylight and artificial light are obtainable. See also Indicators, pp. 22 to 24.

Photometers

As defined above, photometers are colorimeters in which the illumination is by light approximately monochromatized by glass light

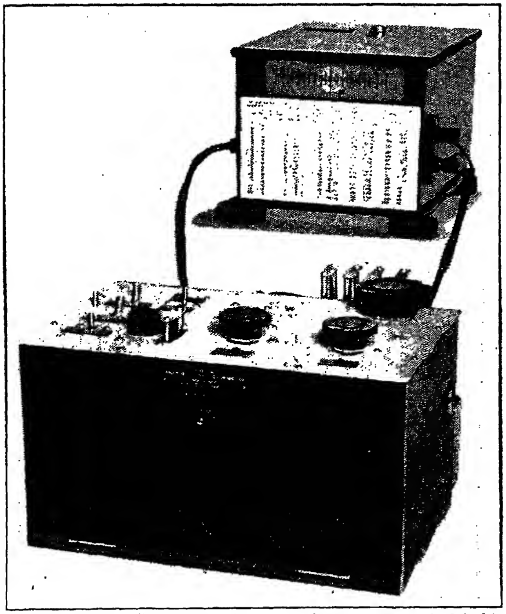


Courtesy of Klett Manufacturing Co., New York

Fig. 11. Klett-Summerson Photoelectric Colorimeter with Transformer and Galvanometer.

filters which should not be confused with the standard matching slides or disks. Examples of the visual type are *Zeiss-Pulfrich Photometer* and *Aminco Neutral Wedge Pho-*

tometer (American Instrument Company, Silver Spring, Maryland); of the photoelectric type, *Aminco Type F Photometer, Klett-Summerson Photoelectric Colorimeter* (Klett Manufacturing Company, New York), *Evelyn Photoelectric Colorimeter* (Rubicon Company, Philadelphia), and *Lumetron Photoelectric Colorimeter* (Photovolt Corporation, New York). (Figs. 11 and 12.)



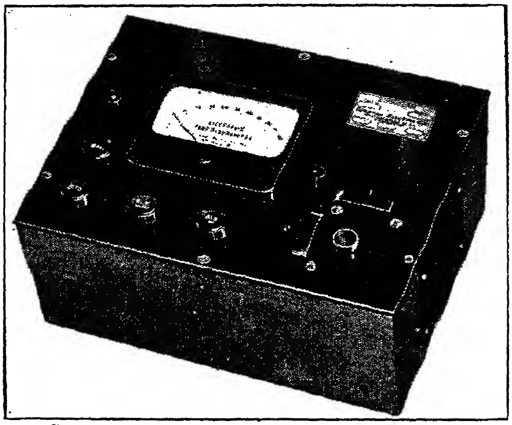
Courtesy of Rubicon Company, Philadelphia

FIG. 12. Evelyn Photoelectric Colorimeter.

The accurately adjusted standard color filters represent definite narrow bands of the visual spectrum and each is designated according to the principal wave length it transmits.

FLUOROMETERS

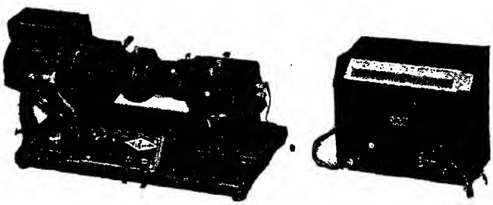
Examples of fluorometers are the *Coleman Photofluorometer* (Fig. 13), the *Klett Fluorimeter*, and the *Pfaltz & Bauer Fluorophotometer* (Fig. 14). The *Coleman Universal Spectrophotometer* may be converted into a fluorometer by the addition of the *Universal Ultra-*



Courtesy of Coleman Electric Co., Inc., Maywood, Ill.

FIG. 13. Coleman Photofluorometer.

Violet Illuminator. The *Lumetron* (Photovolt Corporation, New York) may be used either for fluorescence or colorimetric measurements if the attachments are changed.



Courtesy of Pfaltz & Bauer, Inc., New York

FIG. 14. Pfaltz & Bauer Fluorophotometer.

SPECTROPHOTOMETERS

In instruments of the spectrophotometer class, approximate monochromatization is secured by diffraction gratings or glass prisms, which replace glass color filters. Any desired part of the visible spectrum may be selected. The absorption or transmission of

Microscopes and Accessories

Microscopes are classed as simple and compound, depending on the construction. A simple microscope can be used only for low magnification. High magnification is secured by the combination of eyepiece and objective of the compound instrument.

Simple Microscope. A *lens*, or simple combination of lenses, supplemented by a suitable *support*, a *focusing device*, a *stage*, and an *illuminating mirror*, is known as a simple or dissecting microscope. Its range of magnification is from 7 to 20 diameters. It is suited for a preliminary survey of raw and comminuted vegetable and animal powders, natural and synthetic crystals, and other uses preliminary to mounting for examination with the compound microscope.

Compound Microscope. The fundamental difference between a simple and a compound microscope is that the former produces magnification by a single lens or a compact combination of lenses and the latter by an *objective* at the lower end of two *telescope tubes* and an *eyepiece* (ocular) at the top, each being interchangeable with other objectives and eyepieces, thus securing a wide range of magnification. By multiplying the magnification given by the manufacturer for the eyepiece by that for the objective, the total magnification at standard tube length is obtained; it can be increased or decreased by lengthening or shortening the tube. A multiple *nosepiece* adds to the convenience.

Low Power Instrument. When a considerable amount of work requiring low magnification but a wide field is carried on, the substitution, for a dissecting microscope, of a binocular low power compound microscope reduces the eye strain. By suitable combinations of objectives and eyepieces of different powers, a range in magnification from less than 2.5 to over 200 diameters is possible, but a narrower range, such as 3.5 to 40, is more practicable.

High Power Instrument. High and low power are relative terms, since the range in the two types overlaps.

In addition to the simple equipment of the low power instrument, a *fine adjustment mechanism* and a *substage condenser* for accurate adjustment of the light are essential. Other desirable attachments are a *mechanical stage* and a *polarizing combination* consisting of a *polarizer* in the substage and an *analyzer* in the tube or over the eyepiece.

Either the square-stage biological microscope with mechanical stage or the round-stage chemical or crystallographic microscope with polarizing apparatus is suited for general work in food microscopy, but a combination of the two, with an *oil immersion objective*, *round stage*, *removable mechanical stage*, *substage condenser*, and *removable polarizing apparatus*, is highly desirable. If not provided originally, the attachments may be added later as needed.

Microscopic Accessories. Apart from the microscope and its attachments, the following are essential: *slides* (3 x 1 in.), *cover glasses* (No. 2 ¾-in. circles, for ordinary work), *needles*, *delicate forceps*, *small scalpels*, *reagent bottles*, and *Gilette-type razor blades* for cutting sections.

Microtome. For work requiring uniformly thin and neatly cut sections, especially if in series, the microtome is essential. *Baths* for paraffin and celloidin embedding and other equipment are described in works on vegetable and animal histology.

Drawing and Photographic Apparatus. A *camera lucida* attached to the tube of the compound microscope, with suitable adjustment of light on the object and reflected from the drawing paper at the side of the microscope into the eye, enables the observer to see pencil and object at the same time and trace essential parts of a field without including irrelevant and confusing details. The camera lucida manufactured by the Spencer

Lens Company is so constructed as to overcome certain defects of earlier forms.

The *photomicrographic camera* serves for recording clearly all parts of a field in focus and less clearly those parts out of focus.

Drawings vs. Photomicrographs. Both camera lucida drawings and photomicrographs have their special provinces. Drawings representing study of the specimens, combined with one's knowledge and experience, are invaluable in investigations in the realms of both pure and applied (including forensic) science. By focusing, several superimposed tissues may be drawn. For didactic purposes, carefully elaborated and reproduced drawings made with the aid of the camera lucida have long been preeminent.

Photomicrographs can be taken by anyone skilled in photography and unskilled in science. The camera does not select or interpret. To say that it cannot lie is a mistake. It reduces all colors to shades of one, usually black, and portrays all opaque objects as dark and structureless regardless of the color or detail. It distorts objects out of focus. It does not eliminate air bubbles or extraneous matter. On the other hand, the photomicrographic camera makes satisfactory pictures of certain well-defined bodies, such as starch grains, and of accurately cut and carefully cleared and stained sections. It also is used to advantage in obtaining a general view of delicate and intricate structures, drawings of which would require labor and skill disproportionate to the results.

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[1] Wiley: *Am. Fertilizer* Feb. 7, 1925; *Ind. Eng. Chem.* 1925, **17**, 304.

[2] *J. Ind. Eng. Chem.* 1914, **6**, 78.

[3] Palkin, Murray, and Watkins: *Ind. Eng. Chem.* 1925, **17**, 612.

[4] *Principles and Practice of Chromatography*, translated by Bacharach and Robinson, New York, 1941.

[5] *Physical and Chemical Methods of Sugar Analysis*, New York, 3rd Ed., 1941.

[6] *The Polarographic Method of Analysis*, Easton, Pa., 1941.

Unless otherwise stated, all reagents are chemically pure (reagent grade); acids, ammonium hydroxide, and alkali-metal hydroxides are concentrated; and water, in addition to being distilled, is specially purified if that is deemed

Under the head *Reagents* in the description of each method are included those requiring special preparation, to avoid breaks in the continuity of the directions for the manipulation. By the simple expedient of italicizing all reagents as named under *Process* the consecutive steps in the procedure are clearly indicated.

Concentration. The strength or dilution of solutions, following the system of the originator of a method, is commonly expressed either (1) as parts by weight or volume of the solid or liquid chemical plus parts by volume of the solvent or diluent added, which is water if not otherwise stated, or (2) as percentage, meaning grams or milliters of the chemical dissolved or mixed with the solvent or diluent and made up to 100 ml.; thus 1 + 3 sodium sulfate and 1 + 3 sulfuric acid are equivalent to 25% sodium sulfate and 25% sulfuric acid respectively. Obviously percentages by weight and by volume of liquids are not the same and the expression "grams per 100 ml." is not equivalent to true percentage, but the exact statement of the concentration of a reagent, when not important, is sacrified for brevity. Normality or molar concentration is not usually given except for standard solutions.

Nomenclature. The term *ethanol* is used in place of alcohol, ethyl alcohol, or grain alcohol, the strength other than 95% being always given; *methanol* is used in place of methyl alcohol, wood alcohol, etc.; and so on with *propanol, butanol,* etc.; but the term *amyl alcohol* is retained.

Naphtha, which is listed in *The Merck Index* as a synonym for petroleum ether and benzine, has been chosen as being the most distinctive of the three terms. It cannot be confused with benzene when spoken and is not, like petroleum ether, a misnomer. It was adopted after the perusal of hundreds of papers with such inexact terms as "both ethers" and "mixed ethers" (referring to naphtha and ethyl ether) or the name of the rectifier which means nothing to a foreigner today or an American tomorrow. The statement of the boiling point is desirable, but is not usually given by authors.

Shelf Reagents

The following reagents include commonly used reagent-grade acids, alkalies, solvents, salts, saline solutions, and special reagents. *Unless otherwise stated, individual mineral acids, acetic acid, also ammonium, sodium, and potassium hydroxides, are concentrated and ethanol is 95%.* Concentrations other than those given herewith are indicated under the individual methods. Additional reagents are listed in *The Merck Index,* either by the chemical substance or by the author's name.

Acetic Acid, $CH_3\cdot COOH$, glacial, sp.gr. 1.049 at 25°.

Acetic Anhydride, $(CH_3\cdot CO)_2O$.

Acetone, $CH_3\cdot CO\cdot CH_3$.

Alcohol. See Ethanol.

Alumina Cream, $Al(OH)_3$. Add with stirring to a saturated alum solution ammonium

hydroxide to alkaline reaction, wash the precipitate thoroughly by decantation, decant the clear liquid, and store the suspension.

Ammonium Acetate, $NH_4C_2H_3O_2$, 1 + 10 solution.

Ammonium Carbonate Solution. Dissolve 1 part of the salt (ammonium bicarbonate + ammonium carbamate) in a mixture of 4 parts of water and 1 part of ammonium hydroxide.

Ammonium Hydroxide, sp.gr. 0.90.

Ammonium Molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. See Part I, C8, Phosphorus.

Ammonium Nitrate, NH_4NO_3, powder.

Ammonium Sulfide, yellow, $(NH_4)_2S$, 16 to 20% solution.

Ammonium Thiocyanate, NH_4SCN, 1 + 10 solution.

Amyl Alcohol, $C_5H_{11} \cdot OH$.

Barfoed Reagent. See Part I, C6*a*, Carbohydrates.

Barium Chloride, $BaCl_2 \cdot 2H_2O$, 1 + 10 solution.

Barium Hydroxide, $Ba(OH)_2$, 1 + 20 solution and solid.

Benedict Reagent. See Part I, C6*a*, Carbohydrates.

Biuret Reagent. See Part I, C4*a*, Proteins.

Bromine.

Bromine Water, saturated.

Butanol (Butyl Alcohol), $C_4H_9 \cdot OH$.

Calcium Carbonate, $CaCO_3$, powder.

Calcium Chloride, $CaCl_2$, anhydrous, granular.

Calcium Hydroxide, $Ca(OH)_2$, saturated solution and powder.

Calcium Oxide, solid.

Carbon Dioxide, CO_2, solid, also gas from generator or cylinder.

Carbon Tetrachloride, CCl_4.

Carr and Price Antimony Trichloride Reagent. See Part I, C10, Vitamin A.

Chlorine Gas. Use from generator or cylinder.

Chlorine Water.

Chloroform, $CHCl_3$.

Chlorzinc Iodine Solution. See Part I, A.

Cupric Oxide, CuO, powder.

Cupric Sulfate, $CuSO_4 \cdot 5H_2O$, 1 + 10 solution.

Diammonium Phosphate, $(NH_4)_2HPO_4$. Add NH_4OH to diluted H_3PO_4 until neutral.

Disodium Phosphate, $Na_2HPO_4 \cdot 12H_2O$, 1 + 10 solution.

Ethanol (Ethyl Alcohol), $C_2H_5 \cdot OH$. Used without qualification, 95% by volume is meant. Other percentages by volume and absolute (anhydrous) are stated.

Ether (Ethyl Ether), $(C_2H_5)_2O$, U.S.P. and anhydrous.

Ethyl Acetate, $CH_3 \cdot CO_2 \cdot C_2H_5$.

Fehling-Allihn Copper Reagent. See Part I, C6*a*, Dextrose, Allihn Method.

Fehling-Soxhlet Copper Reagent. See Part I, C6*a*, Reducing Sugars, p. 171.

Ferrous Ammonium Sulfate (Mohr's Salt), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$.

Formaldehyde, HCOH, 40%.

Halphen Reagent. See Part II, B2, Cottonseed Oil.

Hanuš Iodine Solution. See Part II, B2, Iodine Number, Hanuš Method.

Hopkins and Cole Glyoxylic Acid Reagent. See Part I, C4*a*.

Hydrochloric Acid, sp.gr. 1.184.

Hydrogen Gas. Use from generator or cylinder.

Hydrogen Sulfide Gas, H_2S. Use from generator.

Iodine in Potassium Iodide Solution, 1 + 4 + 300.

Iodine Solution (Chlorzinc). See Part I, A.

Iron, by hydrogen, powder.

Lead Acetate (normal), $Pb(C_2H_3O_2)_2 \cdot 3H_2O$, 1 + 10 solution.

Lead Nitrate, $Pb(NO_3)_2$, crystals.

Lead Subacetate, $Pb(C_2H_3O_2)_2 \cdot Pb(OH)_2$. See Part II, E2, Sucrose, Clerget Method.

Magnesia Mixture. See Part I, C8*a*, Phosphorus.

Magnesium Acetate, $Mg(C_2H_3O_2)_2 \cdot 4H_2O$, 4.654 + 1000 solution.

Magnesium Oxide, MgO, powder.

Mercuric Acetate, $Hg(C_2H_3O_2)_2$, 1 + 4 solution.

Mercuric Chloride, $HgCl_2$, 1 + 16 and saturated solutions.

Mercuric Nitrate, $Hg(NO_3)_2 \cdot H_2O$, 1 + 10 solution.

Mercuric Oxide, red, HgO, powder.

Mercurous Nitrate, $HgNO_3 \cdot H_2O$, 1 + 16 solution.

Methanol (Methyl Alcohol), $CH_3 \cdot OH$.

Millon Mercuric Nitrate-Nitrite Reagent. See Part I, C4*a*, Millon Test.

Naphtha (Petroleum Ether, Benzine, Gasoline).

Nitric Acid, sp.gr. 1.40 at 25°.

Nitric Acid, fuming, sp.gr. 1.50.

Nitrogen Gas. Use from generator or cylinder.

Oxygen Gas. Use from generator or cylinder.

Phloroglucinol, $C_6H_6O_3 \cdot 2H_2O$. Dissolve 11 g. in 1500 ml. of 12% hydrochloric acid.

Phosphoric Acid, H_3PO_4, 85%.

Phosphotungstic Acid, $20WO_3 \cdot 2H_3PO_4 \cdot 25H_2O$, 20% solution. (See also p. 73.)

Picric Acid, $C_6H_2(OH)(NO_2)_3$.

Platinic Chloride, $H_2PtCl_6 \cdot 6H_2O$, 1 + 4 solution. If made from the metal, dissolve in aqua regia, evaporate, add 2 portions of hydrochloric acid, evaporating after each, then dilute so that 10 ml. = 1 g. platinum.

Potassium Dichromate, $K_2Cr_2O_7$, 1 + 10 solution and crystals.

Potassium Ferricyanide, $K_3Fe(CN)_6$, 1 + 10 solution.

Potassium Ferrocyanide, $K_4Fe(CN)_6 \cdot 3H_2O$, 1 + 12 solution.

Potassium Hydroxide, KOH, solid and in solutions.

Potassium Permanganate, $KMnO_4$, crystals.

Potassium Sulfate, K_2SO_4, powder.

Potassium (or Sodium) Sulfide, K_2S (or Na_2S), 4%.

Propanol (Propyl Alcohol), $C_3H_7 \cdot OH$.

Silver Nitrate, $AgNO_3$, 1 + 20 solution.

Soda Lime, granular.

Sodium Acetate, $NaC_2H_3O_2 \cdot 3H_2O$.

Sodium Bicarbonate, $NaHCO_3$, powder.

Sodium Bisulfite, $NaHSO_3$, 1 + 4 solution, freshly prepared.

Sodium Carbonate, Na_2CO_3, powder and 1 + 5 solution.

Sodium Cyanide, $NaCN$, 5% solution.

Sodium Hydroxide, $NaOH$, solid and in solutions.

Sodium Hyposulfite (Sodium Hydrosulfite, Merck), $Na_2S_2O_4$, powder; not to be confused with sodium thiosulfate (hyposulfite of photography).

Sodium Nitrite, $NaNO_2$, 3 + 10 solution.

Sodium Sulfate, Na_2SO_4, anhydrous, powder.

Sodium Thiocyanate, $NaSCN$, 1 + 10 solution.

Sodium Thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, powder.

Stannous Chloride, $SnCl_2 \cdot 2H_2O$, 40% in hydrochloric acid.

Sulfur Dioxide Gas, SO_2. Use from generator or cylinder.

Sulfuric Acid, sp.gr. 1.84.

Sulfuric Acid, Babcock, sp.gr. 1.82 to 1.83 at 20°.

Tillmans-Hirsch Indophenol Reagent. See Part I, C10, Vitamin C.

Trichloroacetic Acid, $CCl_3 \cdot COOH$.

Tungstic Acid, H_2WO_4, 10% solution.

Uranium Acetate, $UO_2(C_2H_3O_2)_2 \cdot 2H_2O$, 5% solution.

Wijs Reagent. See Part II, B2, Iodine Number, Wijs Method.

Zinc, granular.

Zinc Dust.

Principal Standard Solutions

Some of the methods for food analysis require special standard solutions, the preparation and standardization of which are described in connection with the other details.

Other methods employ standard solutions long used in general inorganic and organic volumetric analysis. Directions for the preparation of some of these follow.

Standard Acid and Alkali, 0.1 *N*. See Part I, C1*c*, Kjeldahl Method, Willfarth Modification. In addition to or instead of 0.1 *N* ammonium hydroxide, it is desirable to have on hand 0.1 *N* potassium or sodium hydroxide solution, since neither loses strength by evaporation, although they require due precautions against frozen stopcocks and absorption of carbon dioxide. In addition to the indicators suitable for use with standard ammonium hydroxide (cochineal, methyl orange, etc.), phenolphthalein, which is not suitable, gives a decisive end-point with standard potassium and sodium hydroxide.

Standard Sodium Thiosulfate Solution, 0.1 *N*. A. PREPARATION FROM $Na_2S_2O_3 \cdot 5H_2O$. Dissolve 24.82 g. of reagent-grade sodium thiosulfate crystals in recently boiled and cooled water and dilute to 1 liter in a volumetric flask.

Standardization. Titrate, as directed in Part II, B2, under Iodine Number, Hanuš Method, (1) against about 0.2 g. of resublimed iodine in 10 ml. of 15% potassium iodide solution and, as a check, (2) against 20 ml. of 0.1 *N* potassium dichromate solution, to which have been added 10 ml. of 15% potassium iodide solution and 5 ml. of hydrochloric acid. Dilute in both cases with recently boiled and cooled water and add 1 ml. of 0.5% starch indicator after the yellow color has almost disappeared.

B. PREPARATION FROM ANHYDROUS $Na_2S_2O_3$.[1] Dehydrate the crystallized salt first over sulfuric acid until the crystals are transformed into a powder that does not fuse when heated in a test tube at 50°, then heat at 80° while stirring. Weigh 15.81 g. of the powder into a 1-liter volumetric flask, dissolve in water, and dilute to the mark at room temperature.

Standardization. Proceed as directed under A.

Standard Iodine Solution, 0.1 *N*. Weigh 20 g. of potassium iodide into a 1-liter volumetric flask, add sufficient freshly boiled and cooled water to dissolve the iodide; then add 12.692 g. of freshly prepared resublimed iodine and rotate the flask until the iodine dissolves, make up to the mark at room temperature, and shake. Standardize against standard 0.1 *N* sodium thiosulfate solution.

If the solution is deficient in strength, add a calculated amount of a solution of greater concentration, say a 0.2 *N* solution prepared in a 100-ml. volumetric flask from one-fifth the quantities of potassium iodide and iodine used above. The solution loses strength on standing and should be restandardized just before use.

Standard Potassium Permanganate Solution, 0.1 *N*. The theoretical amount of permanganate for 1 liter of the standard solution is 3.1606 g., but it is usually recommended to weigh a trifle more to allow for deterioration during the few days of storage, after which time the solution should be accurately adjusted. Filtration through a plug of asbestos is desirable.

Standardization by Iron Wire (Marguerittte). Iron wire with a minimum of 99.8% purity is supplied by Merck & Co.; iron by hydrogen is of lesser purity. The reaction follows:

$$2KMnO_4 + 10FeSO_4 + 8H_2SO_4 \rightarrow$$
$$K_2SO_4 + 2MnSO_4 + 5Fe_2(SO_4)_3 + 8H_2O$$

Proceed as follows. Place 0.2 to 0.25 g. of the iron in a small flask fitted with a Bunsen valve containing 50 ml. of water and 5 ml. of sulfuric acid, boil for several minutes, cool, and titrate with the standard permanganate solution; 1 ml. of standard 0.1 *N* potassium permanganate solution = 0.005585 g. of Fe or 0.007985 g. of Fe_2O_3.

Standardization by Sodium Oxalate (Sørensen).[2] The reaction is as follows:

$$2KMnO_4 + 5Na_2C_2O_4 + 8H_2SO_4 \rightarrow$$
$$K_2SO_4 + 2MnSO_4 + 5Na_2SO_4 +$$
$$10CO_2\uparrow + 8H_2O$$

Proceed as follows. Weigh 0.25 to 0.30 g. of sodium oxalate (U. S. Bur. Standards) and dilute to 40 to 50 ml. Add 12 to 15 ml. of 4 *N* sulfuric acid, heat to 75 to 80°, and, while at that temperature, titrate slowly, while stirring, with the permanganate solution, allowing time for the solution to become colorless after each addition.

CALCULATION. Apply: 1 ml. of 0.1 *N* $KMnO_4$ = 0.0067 g. of $Na_2C_2O_4$.

Standard Silver Nitrate Solution, 0.1 *N*. Dissolve 16.989 g. of clean dry crystals of purest silver nitrate in water and dilute to 1 liter in a volumetric flask. Fresenius directs to fuse the silver nitrate, but this is not necessary now that Merck & Co. and doubtless other manufacturers supply the salt with a minimum purity of 99.9%.

Standardization. As a check, titrate the 0.1 *N* silver nitrate solution against 0.1 *N* sodium chloride solution, prepared by dissolving in water 5.845 g. of the salt, previously heated below redness and cooled, and diluting to 1 liter. Use 1 ml. of 5% potassium chromate as indicator.

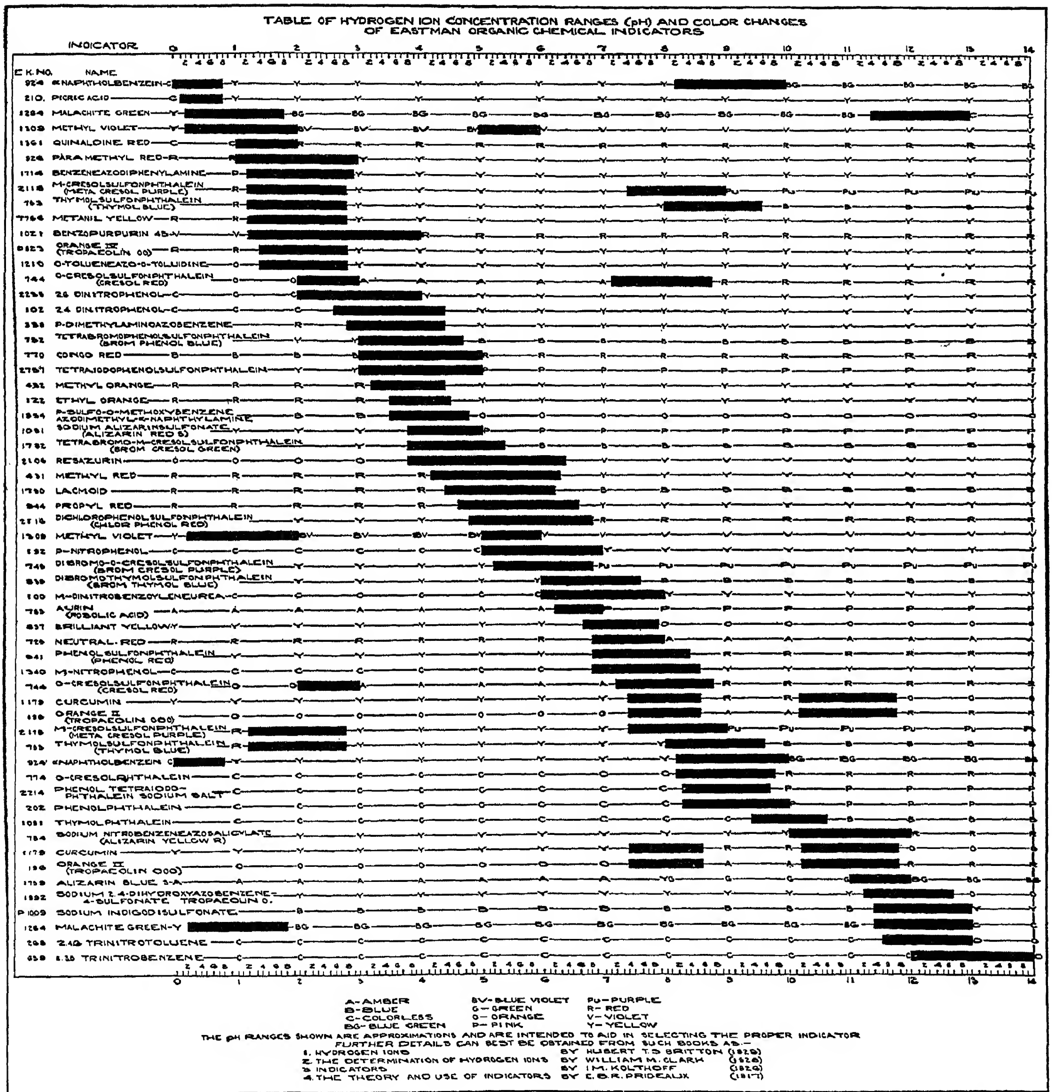
NOTE. For use in the Volhard method, prepare also a 0.1 *N* ammonium or potassium thiocyanate solution, standardized against the 0.1 *N* silver nitrate solution, using saturated ferric alum as indicator. It is convenient to prepare for use in the Mohr method a standard solution containing in 1 l. 4.7919 g. of silver nitrate instead of 16.989 g.; 1 ml. = 1 mg. of chlorine. (Both volumetric methods are described in Part I, 8*a*, Chlorine.) For use in the determination of chlorine in water, brine, and salt, in the *Methods of Analysis* of the A.O.A.C. instructions are given to prepare the potassium chromate solution by dissolving 5 g. of the chromate in water, adding concentrated silver nitrate solution until a slight permanent precipitate forms, then filtering, and diluting to 100 ml.

INDICATORS

Organic *p*H Indicators. The accompanying table shows the hydrogen ion concentration ranges (*p*H) and the color changes of Eastman organic chemical indicators.

***p*H Indicator Solutions.** *Eastman Formulas.* The following formulas are suggested by the Eastman Kodak Company. *Alizarin blue S*, ethanol.* *Aurin*, 0.04 g. in 40 ml. ethanol + 60 ml. water. *Benzeneazodiphenylamine*, 0.01 g. in 1 ml. *N* HCl + 50 ml. ethanol + 49 ml. water. *Benzopurpurin* 4B, 0.1% in water. *Congo red*, 0.1% in water. *o-Cresolphthalein*, 0.04% in ethanol. *Curcumin*, ethanol. *p-Dimethylaminoazobenzene*, 0.1 g. in 90 ml. ethanol + 10 ml. water. *m-Dinitrobenzoyleneurea*, 25 g. in 115 ml. *M* NaOH + 500 ml. boiling water, filtered and cooled; 0.292 g. of the salt in 100 ml. water. *2,4-Dinitrophenol*, saturated water solution. *2,6-Dinitrophenol*, 0.05% in water. *Ethyl orange*, 0.05 to 0.2% in water or aqueous ethanol. *Lacmoid*, 0.2% in ethanol. *Malachite green*, water. *Metanil yellow*, 0.01% in water. *Methyl orange*, 0.01% in water. *Methyl red*, 0.02 g. in 60 ml. ethanol + 40 ml. water. *Methyl violet*, 0.01 to 0.05% in water. *a-Naphtholbenzein*, 1% in dilute alkali. *Neutral red*, 0.01 g. in 50 ml. ethanol + 50 ml. water. *m-Nitrophenol*, 0.3% in water. *p-Nitrophenol*, 0.1% in water. *Orange II*, 0.1% in water. *Orange IV*, 0.01% in water. *Para-methyl red*, ethanol. *Phenolphthalein*, 0.05 g. in 50 ml. ethanol + 50 ml. water. *Phenoltetraiodophthalein sodium salt*, water. *Picric acid*, water. *Propyl red*, ethanol. *Quinaldine red*, 1% in ethanol. *Reazurin*, water. *Sodium*

* In all cases 95%.



Courtesy of Eastman Kodak Company, Rochester, N. Y.

alizarinsulfonate, dilute solution in water. *Sodium 2,4-dihydroxyazobenzene-4'-sulfonate,* 0.01% in water. *Sodium indigodisulfonate,* water. *Sodium nitrobenzeneazosalicylate,* 0.01% in water. *p-Sulfo-o-methoxybenzeneazodimethyl-a-naphthylamine,* 0.1% in 60% ethanol. *Thymolphthalein,* 0.04 g. in 50 ml. ethanol + 50 ml. water. *o-Tolueneazo-o-toluidine,* water. *1,3,5-Trinitrobenzene,* 0.1 to 0.5% in ethanol. *2,4,6-Trinitrotoluene,* 0.1 to 0.5% in ethanol. *2-Amino-5-azotoluene,* see *o-Tolueneazo-o-toluidine.*

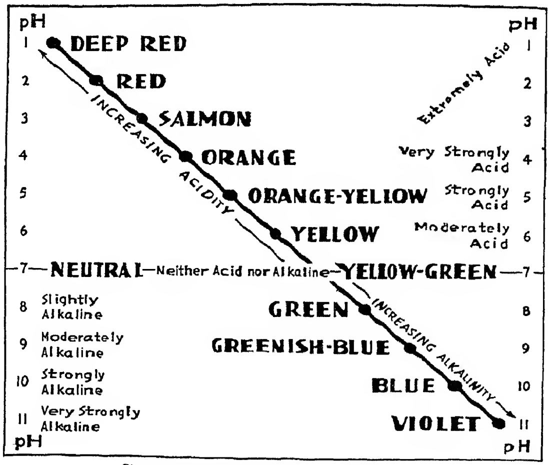
Prepare 0.04% solutions of the sulfonphthalein-type indicators by grinding 0.1 g. of the dry dye, supplied in the acid form, with the number of milliliters of 0.01 N sodium hydroxide solution indicated in the following table to form the sodium salt, and dilute to 250 ml.

o-Cresolsulfonphthalein (cresol red)	26.2
m-Cresolsulfonphthalein (*m*-cresol purple)	26.2
Dibromo-*o*-cresolsulfonphthalein (bromocresol purple)	18.5
Dibromothymolsulfonphthalein (bromothymol blue)	16.0
Dichlorophenolsulfonphthalein (chlorophenol red)	23.6
Phenolsulfonphthalein (phenol red)	28.2
Tetrabromo-*m*-cresolsulfonphthalein (bromocresol green)	14.3
Tetrabromophenolsulfonphthalein (bromophenol blue)	14.9
Tetraiodophenolsulfonphthalein	11.7
Thymolsulfonphthalein (thymol blue)	21.5

The Eastman Kodak Company also supplies a universal mixed indicator that in passing from pH 4 to 10 gives a series of colors through the whole range of the spectrum with an accuracy within 0.5 pH, thus: pH 4.0 red, 4.5 orange-red, 5.0 orange, 5.5 orange-yellow, 6.0 yellow, 6.5 yellow-green, 7.0 pure green, 7.5 green (slightly bluish), 8.0 green-blue, 8.5 blue-green, 9.0 blue, 9.5 blue-violet, and 10.0 violet.

Hellige Indicators. The pH indicator solutions, for which glass standards are supplied (see Hellige Comparator under Apparatus, Colorimeters, above), are cresol red-A 0.2 to 1.8, thymol blue-A 1.2 to 2.8, methyl violet-D 1.8 to 3.2, dinitrophenol-B 2.2 to 3.8, dinitrophenol-A 2.8 to 4.4, bromophenol blue-D 3.0 to 4.6, dinitrophenol-G 4.0 to 5.4, bromocresol green-D 4.0 to 5.6, methyl red-D 4.4 to 6.0, chlorophenol red-D 5.2 to 6.8, bromothymol blue-D 6.0 to 7.6, phenol red-D 6.8 to 8.4, cresol red-B 7.2 to 8.8, thymol blue-B 8.0 to 9.6; cresol phthalein-D 8.4 to 9.8, phenol phthalein-D 8.6 to 10.2, thymolphthalein-D 9.2 to 10.6, purple-D (applicable in the presence of lime salts) 10.0 to 11.4, nitro yellow-D (not applicable in the presence of lime salts) 10.0 to 11.6, alizarin-D 10.2 to 12.0, tropaeolin-D 11.2 to 12.8, and violet-D 12.0 to 13.6.

The Hellige wide range indicator (pH 1 to 11) yields colors in approximately the order



Courtesy of Hellige, Inc., Long Island City, N. Y.

FIG. 17. Hellige Wide Range Indicator Graph.

in the spectrum, as shown in the graph (Fig. 17), reproduced through the courtesy of Hellige, Inc.

RESULTS

Examples. In the early pages of some of the chapters and subchapters of Part II are given tables of analyses of typical products and also, after the description of the individual methods, selected results if such are available in the original paper. The meager figures reported serve chiefly for the general guidance of the reader as to the approximate amount of the principal constituents, such as fat, protein, starch, and sugars, or minor constituents, such as trace elements, vitamins, and flavors, as well as in some cases for the comparison of results by different methods.

Compilations. Those who have need of exhaustive data on composition are referred to König's *Chemie der menschlichen Nahrungs- und Genussmittel*, Atwater and Bryant's *Chemical Compilation of American Food Materials*, Jenkins and Winton's *Compilation of Analyses of American Feeding Stuffs* (the last two published as bulletins of the U. S. Department of Agriculture), and the authors' *Structure and Composition of Foods*. In the compilations named, in addition to analyses of the materials as produced, analyses calculated to the water-free basis are also given.

Abbreviations and Symbols. There have been errors and a lamentable lack of uniformity in the use of symbols. The Greek letter α, which means optical rotation, has often been substituted for $[\alpha]$, which means specific rotation.

The gamma is one-thousandth of a milligram or one-millionth of a gram. The indiscriminate use of γ/g. (gammas per gram), mg./k. (milligrams per kilo), and p.p.m. (parts per million), all meaning the same concentration, has been perplexing. In this book preference is given to the symbols γ/g. and γ/ml.

In microscopic measurements, the micron (μ) is one-thousandth of a millimeter (0.001 mm.), the millimicron (mμ) is one-thousandth of a micron. Certain authors have given micromicron ($\mu\mu$, 0.000,000,001 mm.) when millimicron (mμ, 0.000,001 mm.) was meant.

REFERENCES

[1] Young Method: *J. Am. Chem. Soc.* 1904, **26**, 1028; Treadwell-Hall: *Analytical Chemistry*, New York, 9th Ed. 1942, **2**, 591.

[2] *Z. anal. Chem.* 1903, **42**, 352, 512; 1906, **45**, 272; Treadwell-Hall: *Analytical Chemistry*, New York, 9th Ed. 1942, **2**, 538; *Methods of Analysis*, A.O.A.C.

PART I

GENERAL METHODS

A. GENERAL MICROSCOPIC METHODS

Food microscopy is histology, both vegetable and animal, applied to food products. It has been developed as both a pure and an applied science. As a pure science, it is closely related to anatomy, physiology, and physiological chemistry; all four are treated together in textbooks on biology. As an applied science, it ranks with food chemistry, each contributing its part and supporting the other in problems affecting agriculture, nutrition, medicine, industry, and inspection.

Scope of Food Microscopy. At first food microscopy was largely developed, as stated on the title page of the authors' *Microscopy of Vegetable Foods*, with special reference to the adulteration of foods and the diagnosis of mixtures, but now that fraudulent practices have been curbed, owing to law enforcement and higher commercial ethics, the application is chiefly in other fields. It is used in connection with the control of fungus and insect diseases to show the parts affected, to trace the progress of manufacturing processes, culinary operations, and digestion in the animal body, to study growth, ripening, deterioration, and decay, and above all to place in the hands of the intelligent producer, manufacturer, and consumer a tool for a more thorough understanding of the products, with which they are vitally concerned, for such application as occasion demands. As the writers have had opportunity to demonstrate, in certain problems involving patents and copyrights, where chemical analyses failed, the microscope furnished the desired evidence.

Relation of Food Microscopy to Chemical Analysis. Since the microscopical characteristics of the aleurone (protein) grains, fat or oil, starch grains, cellular elements of the fiber, and certain ash constituents, notably calcium oxalate, calcium carbonate, and sand, are more or less marked in sections cut from the original unground products, microscopic examination alone goes far toward giving an idea of the composition of the material without recourse to chemical analysis. Furthermore, although grinding and processing disrupt the continuity of structure and may destroy the characteristic form of certain elements, enough remains to furnish valuable information, especially if supplemented by the senses of sight, smell, taste, and touch. Starch grains, it is true, are distorted or destroyed by boiling or baking, but they or the paste formed with water still responds to the iodine test.

Microscopic evidence is usually direct; chemical evidence is indirect, requiring interpretation. Often it seems like magic to the layman that a glance at a mount of a portion of each material only a fraction of the head of a pin in bulk enables one to differentiate a large group of white powders. Even percentages may often be estimated roughly, just as an expert in scanning a field of grain may judge with surprising accuracy the bushels per acre.

Introductory Matter. The list of reagents and the brief descriptions of the typical histological elements below, also the matter at the beginning of several of the chapters of Part II, are designed as an introduction to a subject of absorbing interest to which the writers have given the best years of their lives. It should be noted that the space occupied by individual tissue elements in the illustrations does not represent the proportion of such elements in the natural product, since the minor constituents often require a proportionally larger space for their adequate portrayal.

The subject is treated in greater detail in the *Structure and Composition of Foods.* (See page facing title page.)

Apparatus. See Introduction, Apparatus, Microscopes and Accessories.

Reagents. Although histologists have proposed many reagents, especially for staining and preparing permanent slides of embryological specimens, the analyst requires but a few to clear, defat, macerate, stain, and temporarily mount food tissues. Often water alone is all that is necessary. The following list is sufficient for practical work:

Acetic Acid, 1 + 1 glacial acid.

Chloral Hydrate Solution. Dissolve 8 g. of chloral hydrate in 5 ml. of water.

Chlorzinc Iodine Solution. Dissolve 30 g. of zinc chloride, 5 g. of potassium iodide, and 0.89 g. of iodine in 14 ml. of water. Keep a few crystals of iodine in the bottle.

Ethanol.

Ether.

Ferric Chloride Solution, 1%.

Glycerol, 1 + 1.

Iodine in Potassium Iodide Solution. Dissolve 0.05 g. of iodine and 0.2 g. of potassium iodide in 15 ml. of water.

Labarraque's Solution or *Javelle Water.*

Safranin Solution. Dilute a water solution as needed.

Schultze's Macerating Solution. Mix a few crystals of potassium chlorate with nitric acid immediately before using.

Sodium Hydroxide Solution, 5%.

Turpentine.

Structure. All parts of the plant occur in foods: *root* (carrot), *rhizome* (ginger), *tuber* (potato), *stem* (asparagus), *bark* (cinnamon), *leaf* (lettuce), *flower* (broccoli), *fruit* (tomato), and *seed* (shelled legumes).

In cross section the *root* and *stem* show an outer layer (epiderm) separated by the cortex from the central cylinder with its fibro-vascular bundles. *Leaves* have upper and lower epidermal layers separated by masses of chlorophyl-containing tissue. *Flowers,* although complicated in gross structure, are comparatively uninteresting in their histological elements; pollen grains, however, are an exception. *Fruits* are made up of a fruit-coat (pericarp) surrounding the *seed* which has a seed-coat (spermoderm) enclosing the perisperm, the embryo (germ), and the endosperm which may be described as the food for the embryo stored outside its body. The proportion of spermoderm, perisperm, endosperm, and embryo varies greatly. In the cereals only endosperm and embryo are well developed, in the common legumes only the spermoderm and embryo, whereas in black pepper the bulk of the seed is perisperm.

Histological Elements. The more important tissues fall into two classes: cell tissues and cell contents.

I. Cell Tissues. *Collenchyma.* Cells with conspicuous thickenings at the angles; walls non-lignified.

Cork. Protective tissue of tabular cells in radial rows; suberin in the walls.

Cortex. Layer in stems containing the fibro-vascular bundles.

Cuticle. Membranous coating, consisting of cutin, often covering the epiderm.

Emergences. Multicellular outgrowths derived from the epidermal and subepidermal tissues.

Endocarp. Inner layer of fruit-coat, often forming a woody zone.

Epiderm. The outer and inner layers of organs and the upper and lower layers of leaves.

Epicarp. The outer epiderm of the fruit-coat.

Fibro-Vascular Bundles. Compound strands of elongated elements consisting of (1) phloem with sieve tubes, cambiform cells, and parenchyma, (2) xylem with pitted, reticulated, spiral, and annular vessels, and (3) sheath of bast fibers.

Hairs. Unicellular or multicellular outgrowths of the epiderm.

Palisade Cells. Elongated cells arranged vertical to the surface of the organ.

Parenchyma. Thin-walled, isodiametric or moderately elongated cells, with walls usually of cellulose with or without intercellular spaces. Spongy parenchyma has conspicuous intercellular spaces and distorted contour.

Sclerenchyma. Stone cells, fibers, etc., with lignified, often pitted, walls.

Stomata. Epidermal openings that facilitate photosynthesis, respiration, and transpiration.

Water Pores. Openings at the ends of nerves through which water is discharged.

II. Cell Contents. *Aleurone Grains.* Bodies rich in protein consisting of ground substance often containing one or more crystalloids (protein crystals), globoids (believed to consist of lime, magnesia, phosphoric acid, and organic acid), and calcium oxalate crystals.

Calcium Oxalate Crystals, occurring as single monoclinic crystals, crystal rosettes, raphides (needle-shaped crystals), and crystal sand.

Cystoliths, consisting of calcium carbonate.

Inulin, forming spaero-crystals from ethanol.

Latex. Milky secretions in tubes.

Mucilage. Gelatinizing substances.

Protoplasm. The living matter of the cell consisting of granular or stringy cytoplasm, round or oval cell nucleus, and chromatophores such as chlorophyl grains (green bodies), leucoplasts (starch formers), and chromoplasts (yellow, orange, or red bodies).

Starch, occurring as single grains varying greatly in form (globular, lenticular, ellipsoidal, ovoid, truncated, or polygonal) or as aggregates of few or many individuals. Figs. 18 to 29 inclusive show twelve commercial starches; twenty-four others are pictured in *Structure and Composition of Foods* (New York, 1932, I, 28).

Stegmata. Cells containing siliceous bodies.

Sucrose. Visible only when deposited by evaporation.

Tannins, Fats, Fatty Oils, Waxes, Volatile Oils, and *Resins* do not usually occur as definite organized bodies.

LEGENDS OF ILLUSTRATIONS OF COMMERCIAL STARCHES ON FACING PAGE

The magnification is ×160.

FIG. 18. Maize (*Zea Mays* L.). Large grains mostly polygonal (from horny endosperm), less often round (from floury endosperm); diameter up to 30 μ; hilum central, distinct, often with radiating clefts; rings seldom evident.

FIG. 19. Rice (*Oryza sativa* L.). Grains mostly polygonal, present as aggregates in the kernel but largely disintegrated during manufacture; diameter up to 10 μ; hilum central, small.

FIG. 20. Kudzu (*Pueraria hirsuta* Schneider). Grains polygonal, round, or kettledrum-shaped; diameter up to 35 μ; hilum distinct, central or slightly eccentric; rings distinct.

FIG. 21. Cassava (*Manihot utilissima* Pohl and *M. aipi* Pohl). Large grains mostly flask- or kettledrum-shaped; diameter up to 35 μ; hilum usually central, distinct; rings indistinct. Occasional aggregates occur.

FIG. 22 Sweet Potato (*Batatas edulis* Chois.). Large grains kettledrum-shaped, often with more than one truncation; diameter up to 50 μ; hilum eccentric; rings indistinct. Calcium oxalate rosette crystals present.

FIG. 23. Arum (*Arum maculatum* L. and other species). Large grains polygonal or truncated, frequently in small aggregates; diameter up to 22 μ; hilum central with clefts; rings indistinct. Raphides present.

FIG. 24. Canna (*Canna edulis* Edw. and other species). Large grains broadly oval, blunt hilum end narrower than rounded opposite end; diameter up to 145 μ; hilum distinct (sometimes double); rings distinct.

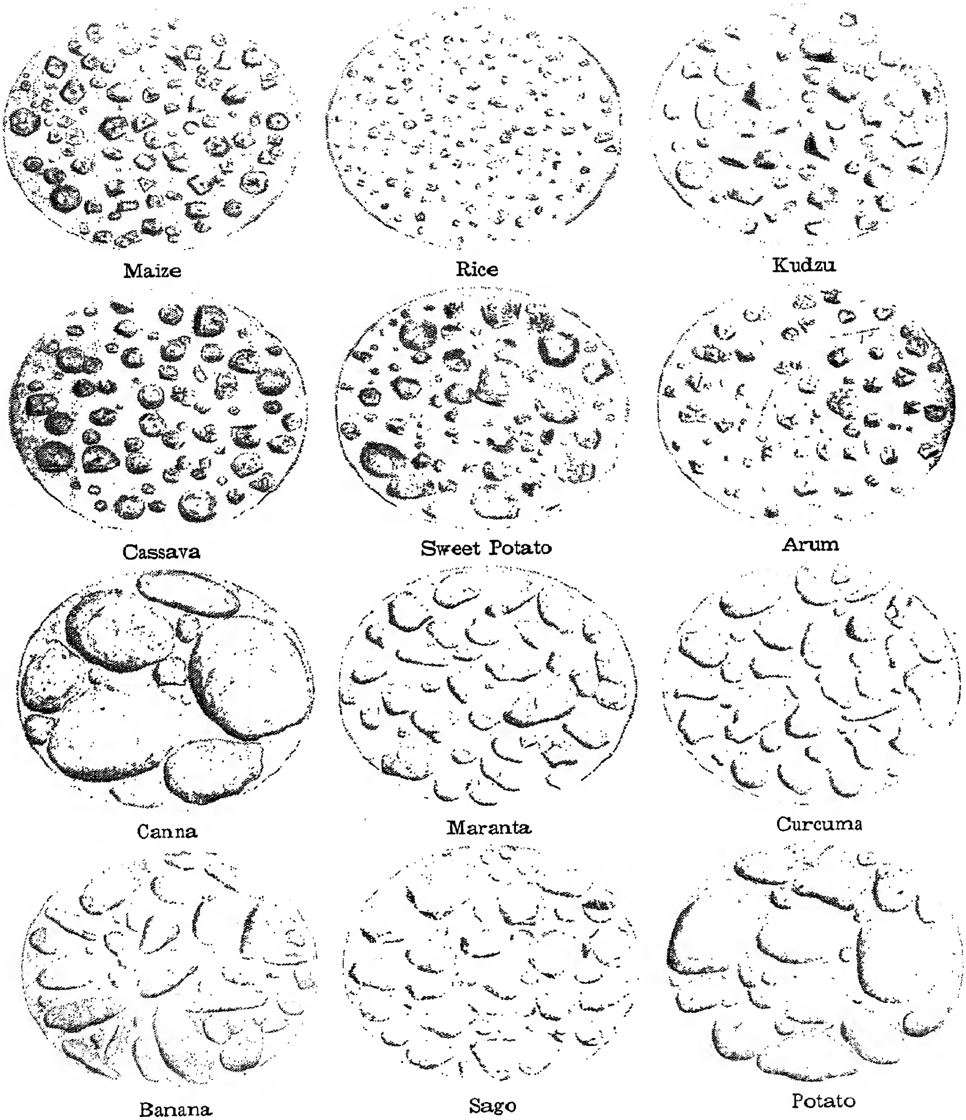
FIG. 25. Maranta (*Maranta arundinacea* L.). Large grains variable, ovoid, spindle-shaped, rounded triangular, etc.; length up to 60 μ; hilum often with fissures suggesting soaring bird; rings distinct.

FIG. 26. Curcuma (*Curcuma angustifolia* Roxb. and other species). Large grains much elongated, pear-, sack-, or club-shaped, narrowed at one end; length up to 75 μ; hilum eccentric; rings distinct.

FIG. 27. Banana (*Musa* sp.). Large grains much elongated, sack-, sausage-, pear-, or club-shaped, narrowed at one end; length up to 85 μ; hilum eccentric, in narrow end; rings distinct.

FIG. 28. Sago (*Metroxylon Rumphii* Mart. and other species). Large grains irregular in shape with truncations; diameter up to 80 μ; hilum in the rounded end, sometimes with clefts; rings distinct. Crystals may be present.

FIG. 29. Potato (*Solanum tuberosum* L.). Large grains ellipsoidal, ovoid, often irregularly lobed, narrow end rounded, opposite end varies; length up to 100 μ; hilum distinct, usually in narrow end. Rings distinct.



FIGS. 18–29. Commercial Starches. (A.L.W.)

Specific Gravity

Apparatus. Any of the forms of apparatus described in the Introduction may be used; each possesses certain advantages. Although all are sufficiently accurate for most purposes, the Sprengel tube is considered especially exact. It is inexpensive and there is little danger of an air bubble vitiating the result. Both ends should have caps.

If a large number of determinations are to be made, the Westphal balance will soon pay for itself in saving of time in both the determination and cleaning. It is accurate and it obviates the danger of a drop falling on the balance pan.

A pycnometer with narrow neck (Fig. 144, I), but without a stopper, combines simplicity with reasonable accuracy. It should be filled through the adapter used as a funnel tube. One with a perforated ground glass stopper may vary in capacity because of irregularity in seating the joint; there is also danger of a drop exuding through the capillary opening in the stopper with a rise in temperature. The calibration should be made with boiled distilled water at the temperature used in the actual determination.

Although it is desirable to make the determination at exactly the desired temperature (15, 25, or 100°C.), the specific gravity may be calculated from results at other temperatures, if the proper correction which, for oils and fats, is 0.0007 for each degree above or below the desired temperature, is used. As such a correction is based by Lewkowitsch on the average of a number of common oils and is not strictly exact for any one oil, it is well to avoid a wide divergence from the conventional temperature.

Refraction

The refractive power of liquids, with or without dilution, and of solids, liquified by heat or in solution, is one of the most important values (constants) determined in foods as a means of diagnosis. For certain products it even ranks ahead of some or all of the other physical values in sharp differentiation and for most products in ease of determination. The chief objection is the expense of the apparatus, two of the three forms being required for conducting all the operations described in this volume. This does not mean that one or both are essential in food analysis, since usually the figures on refraction closely parallel those on specific gravity, and it is doubtful if the instruments render possible practical conclusions that could not be reached by other means. Nevertheless the refractometer greatly facilitates the work of the food analyst.

Apparatus. Of the three instruments, the *Abbé refractometer*, the *butyrorefractometer*, and the *immersion refractometer*, the second is a simplification of the first with a narrower range. It is specially designed for distinguishing imitation butter from the genuine, but serves well for the examination of most oils and fats and certain other substances or their solutions. As in the Abbé instrument, the light passes through a thin film of the sample between two prisms, but the reading is made directly on a scale, whereas in the Abbé refractometer special adjustment is essential before reading the refractive index on a sector. The Abbé instrument is preferable for general work such as the examination of essential oils and the determination of total solids in molasses, sirups, honey, and other saccharine products, whereas the immersion instrument is suited for the detection of water in milk, the determination of ethanol, and the detection of methanol in liquors.

Manipulation of the Abbé Refractometer. Set up the instrument (see Figs. 5 and 6) so

that it will be lighted by reflected sunlight (best in front of a north window) and connect with apparatus for supplying a slow stream of water heated to constant temperature. A tank on a shelf or stand near the instrument, heated by gas or electricity, may be used as a substitute for the apparatus furnished by the instrument maker. For solid fats, employ as the standard temperature 40°; for oils and semi-solid fats, 25°.

The stream of water, after entering the lower prism, runs into the upper prism through a short rubber tube and passes out through the upper tube. When the thermometer shows a constant temperature, as near as possible to the standard of 40 or 25°, turn the milled head until the lower prism drops away from the upper prism. Cover the surface of the lower prism uniformly with a drop or two of the oil or melted fat, close, and turn the milled head until locked. Adjust the mirror and rotate the alidade on the sector until the border line of illumination and shadow appears in the field. Adjust the compensator by means of the milled head until the band of colors changes into a sharp border line. Move the alidade until the border line passes through the intersection of the crossed lines and read the refractive index (n_D) through a lens.

The reading on the compensator may be converted into the mean dispersion by consulting the table accompanying the instrument and calculation.

Temperature Correction. If the temperature varies from the standard temperature (25 or 40°), introduce a correction of 0.000365 for each degree, adding if the observed temperature is the higher and subtracting if the lower. The corrected figure may be obtained without calculation by means of the Leach and Lythgoe slide rule. This rule also serves to convert butyrorefractometer readings into refractive indices.

Manipulation of the Immersion Refractometer. Place in a small beaker the liquid, such as milk serum, liqueur, or saccharine solution, bring to a constant temperature by heating in the special glass-bottom bath, and insert the prism of the instrument into the liquid, using the wire frame for support. Adjust the mirror below the bath so as to throw light through the liquid into the prism and turn the screw of the compensator (if provided) to secure a sharp line of demarcation, then read the line on the scale of the instrument. Also read the exact temperature of the liquid. Correct for temperature, using the appropriate table.

For small amounts of a liquid or for examination out of contact with air, employ a special prism and a small metal beaker attached to the instrument.

FREEZING POINT

The Beckmann apparatus [1] and the Hortvet cryoscope [2] are of particular value in food analysis, especially as a means of detecting added water in milk (Part II, G1).

Hortvet Cryoscopic Method.[2] The details given in Part II, G1, apply not only to milk examination but also to the determination of freezing point of other liquids; for this purpose the Beckmann thermometer (BT) and not the standard thermometer (MT) is employed.

MELTING POINT

See Part II, B2, for the method employing a tube open at both ends. The closed tube method is described in elementary works on organic chemistry.

REFERENCES

[1] *Z. physik. Chem.* **2**, 638.

[2] *J. Ind. Eng. Chem.* 1921, **13**, 198, Personal Communication.

C. GENERAL CHEMICAL METHODS

The groups numbered or lettered as in cross references follow:

NATURAL CONSTITUENTS

1. Organic elements: *a*, carbon and hydrogen; *b*, carbon; *c*, nitrogen.
2. Six constituent groups; *a*, water; *b*, protein; *c*, fat; *d*, nifext; *e*, fiber; *f*, ash.

   All substances present in food logically belong under one of the six heads, numbered below 3 to 8 inclusive; for convenience, however, alcohols, which analytically are included under 3 or 6, are separately classified under 9, and vitamins and colors, logically belonging under 4, 5, and 6, according as each is nitrogenous, ether-soluble, or nitrogen-free and ether-insoluble, are grouped under 10 and 11.
3. Water (loss at the temperature of boiling water).
4. Protein (nitrogenous substances): *a*, pure (true) proteins; *b*, amino acids; *c*, acid amides; *d*, purines and bases; *e*, ammonia; *f*, nitrates.
5. Fat or oil (ether extract): *a*, true fat and fixed oil; *b*, volatile oil.
6. Nifext: *a*, carbohydrates; *b*, organic acids; *c*, tannins, etc.
7. Fiber: *a*, cellulose; *b*, lignin; *c*, cutin; *d*, suberin.
8. Ash (mineral constituents): *a*, principal inorganic elements (silicon, aluminum,* iron,* calcium, magnesium, potassium, sodium, sulfur, phosphorus, chlorine); *b*, minor inorganic elements (so-called trace elements: aluminum,* arsenic, boron, bromine, cobalt, copper, fluorine, iodine, iron,* lead, manganese, selenium, tin, zinc).
9. Alcohols: *a*, ethanol; *b*, methanol; *c*, glycerol; *d*, inositol.
10. Vitamins.
11. Natural colors.

*Classified both as principal and minor inorganic elements.

EXTRANEOUS CONSTITUENTS

12. Artificial colors.
13. Chemical preservatives.

1. ORGANIC ELEMENTS

a. CARBON AND HYDROGEN

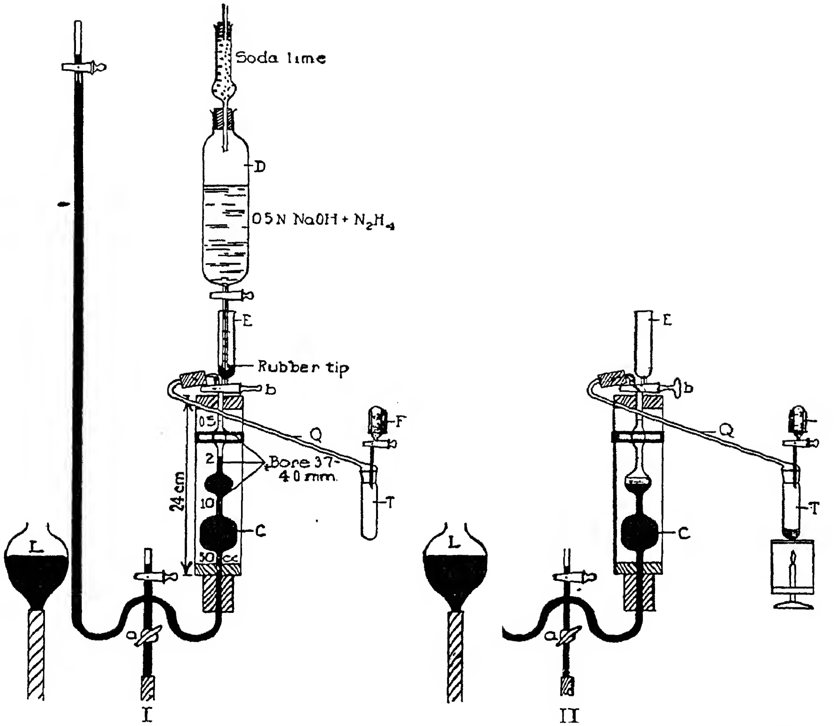
The classical Liebig method for the determination of carbon and hydrogen by combustion with copper oxide and the absorption of the water in calcium chloride or sulfuric acid and of the carbon dioxide in potassium hydroxide solution or soda lime is described in all works on general chemical analysis and on organic analysis. In this work only the more recent Van Slyke and Folch wet combustion method for the determination of carbon in organic substances and several methods for the determination of the carbon dioxide of carbonates are described.

b. CARBON

Van Slyke and Folch Wet Combustion Manometric Method.[1] The oxidation mixture consists of chromic, iodic, sulfuric, and phosphoric acids; the carbon dioxide is absorbed by a solution of sodium hydroxide and hydrazine, then later is liberated by lactic acid.

The method (Rockefeller Institute) is unique among wet combustion carbon methods in that it is accurate, rapid, and, so far as is now known, applicable to all types of organic substances. It is to the Liebig cupric oxide method for carbon (and hydrogen) what the Kjeldahl method is to the Dumas cupric oxide method for nitrogen.

A. Micro and Submicro Combustion. APPARATUS. *Combustion and Manometric Assembly* (Figs. 30 and 31). Except in cer-



Courtesy of the Authors and J. Biol. Chem. 1940, **136**, *512*

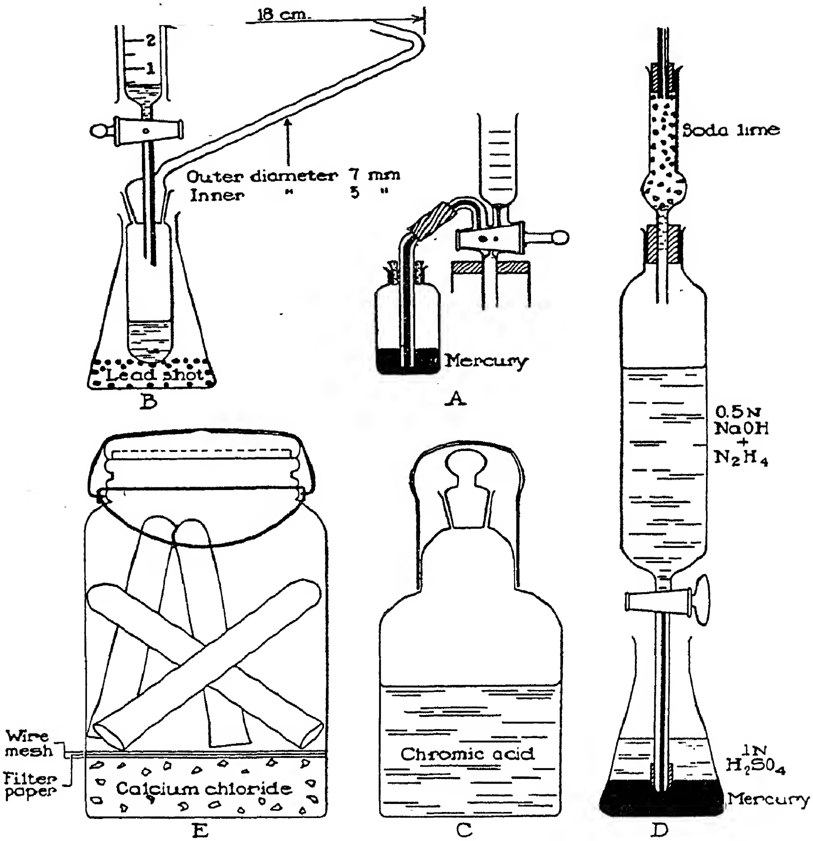
FIG. 30. Van Slyke and Folch Wet Combustion Manometric Assembly.

tain details, the apparatus * is the same as that used by Van Slyke, Page, and Kirk:[2] *T* combustion tube, *D*, *E*, and *F* measuring tubes, *C* manometric chamber, and *L* leveling bulb. *T*, *Q*, and the core of the stopcock below *F* must be of Pyrex glass or its equivalent.

Accessory Apparatus. Stands for *T* and *Q* together and for *D*, when detached from the assembly. *Small bottle* with tube reaching to the bottom for sealing the capillary cock *b* with mercury (Fig. 31, *A*). *Fruit Jar* with layer of calcium chloride for drying and storing combustion tubes (Fig. 31, *E*). *Sheet Aluminum Scoop* (in the shape of a half-cylinder capable of entering a combustion tube) with counterweight and cork stand. *Pincers* with chamois-skin-lined jaws. *Glass Measures* for delivering 100 and 200 mg. of potassium iodide. *Bottle*, with glass stopper and cover, for chromic acid (Fig. 31, *C*).

REAGENTS. *Combustion Reagent.* Place in a 1-liter glass-stoppered Pyrex Erlenmeyer flask 25 g. of chromium trioxide (CrO_3), 167 ml. of sirupy phosphoric acid (sp.gr. 1.7), 333 ml. of fuming sulfuric acid containing 20% of free sulfur trioxide, and 5 g. of potassium iodate. Heat, unstoppered with rotation, until 140 to 150° is reached, then remove the heat, cover with an inverted, lipless beaker, cool to room temperature, stop-

* Obtainable from Eimer and Amend and from Machlett and Son, New York.



Courtesy of the Authors and J. Biol. Chem. 1940, **136**, *612*

FIG. 31. Accessory Apparatus for Van Slyke and Folch Method.

per, and again cover with the inverted beaker. For use, pour a portion of the reagent into a 100-ml. glass-stoppered and covered bottle (Fig. 31, *C*). Moisture must be excluded. Check the strength monthly by mixing 3 ml. of a tenfold dilution with 5 ml. of water and 10 ml. of 10% potassium iodide solution, then, after allowing to stand 5 minutes, titrate with 0.1 *N* sodium thiosulfate solution. If less than 5 ml. of thiosulfate is required, adjust by adding more chromium trioxide.

Alkali-Hydrazine Solution. Acidify 1 or 2 liters of distilled water with a few drops of dilute acid, boil, cool in a flask protected by a soda lime tube, and add clear concentrated (18 to 20 *N*) sodium hydroxide solution to 0.815 *N* as determined by titration. Store in a paraffin-lined aspirator bottle protected by a soda lime tube. Weigh 2 g. of hydrazine

sulfate into a 100-ml. volumetric flask, draw off into the flask through a delivery tube reaching to the bottom the 0.815 N alkali until two-thirds full, shake without withdrawing the tube, fill to the mark, and mix. Transfer by suction about four-fifths of the solution to D. Protect the latter with a soda-lime tube. The hydrazine prevents error from chlorine, bromine, or iodine evolved in the combustion. The hydrazine slowly decomposes in the alkaline solution, about half disappearing in a month; hence renew the alkali-hydrazine solution once a month.

Lactic Acid, 2 N. Dilute c.p. concentrated lactic acid (sp.gr. 1.20) with water to 5 volumes. Check the concentration by titration, using phenolphthalein as indicator.

Sodium Hydroxide Solution, 5 N. Keep in a cylinder such as D in Fig. 31, but with no rubber ring around the delivery tube. Determine the equivalent of 0.5 ml. in number of drops.

PROCESS. (*a*) *Weighing the Charge.* Weigh an amount of a powdered sample, containing 2.5 to 3.5 mg. of carbon, on an aluminum scoop on the left-hand pan of the micro balance, using a counterweight. Lifting the scoop with the pincers, transfer the substance to tube T, placed in a horizontal position, without causing adherence of particles to the walls, and again weigh the scoop. Weigh a hygroscopic substance in a closed tube and a sticky substance in a porcelain boat (15 to 17 mm. long). Slide the container into the tube and drop into the tube a few pieces of *alundum* to insure smooth boiling.

(*b*) *Measuring the Charge in Solution.* When a sub-micro combustion is carried out with 0.3 to 0.7 mg. of carbon, prepare a solution in a suitable solvent containing the desired amount in 1 or 2 ml. and deliver the charge into the combustion tube from an ordinary pipet, or preferably from a weight buret, in either case with a stem reaching to the bottom of the tube.

Leave the tubes overnight somewhat inclined in a large evacuated desiccator. If water is the solvent, or the material is not sensitive to heat, evaporate by heating the lower end of the combustion tube in sulfuric acid contained in a Pyrex beaker heated on a steam bath, protecting the tube from dust. In removing volatile organic solvents add to the tube a few pieces of *alundum* to prevent bumping. Always avoid unnecessary heating and exposure to air. In the thin film deposited in the tube, even relatively stable substances, like glucose and cholesterol, lose carbon measurably by oxidation in an hour on a water bath, or in a day or two at room temperature.

Connection of Combustion Tube with Manometer Chamber. Introduce into the combustion tube (T) from a glass measure 200 mg. of *potassium iodate* for a micro analysis or 100 mg. for a sub-micro analysis. With a medicine dropper draw a ring of *sirupy phosphoric acid* around the upper part of the ground glass joint of T, while in a horizontal position. Into cup F of connecting tube Q, which has been resting in T with which it was last used, pour freshly prepared *combustion reagent* up to the 2-ml. mark and connect with the combustion tube containing the present charge. Connect the latter tube with the manometer chamber (C), which, as shown in Fig. 30, I, is completely filled with mercury.

Preliminary Ejection of Air. Turn cock b to connect C and T and lower the mercury in C to the 50-ml. mark, thereby drawing two-thirds of the air from Q and T over into C. Close cock b, readmit the mercury into C and with cock b open eject the air trapped over the mercury in C through cup E, then again close cock b. By ejecting the air, the blank due to atmospheric carbon dioxide is made insignificant and transfer of carbon dioxide from T to the alkali solution in C is made much more quickly.

Measurement of Alkali-Hydrazine Solution. Open cock b wide and measure 2 ml. of

alkali-hydrazine solution (of the same temperature as water in the jacket) from D into C through the mercury seal, by opening cock a, then closing it when the mercury falls to a level about 1 mm. above the 2-ml. mark. Withdraw D and allow the mercury from E to enter into the chamber to fill the capillary of cock b. The displacement of the slight volume of solution from the capillary into the chamber brings the volume of the solution in C exactly to the 2-ml. mark. Rinse the cup E with *acidified water;* never allow it to stand moistened with alkali.

Combustion. Lower L and draw the mercury out of C and the manometer until the mercury in the latter is about level with the 2-ml. mark on C. Close cock a and turn cock b to connect C with T. Place the leveling bulb as shown in Fig. 30 so that the mercury surface is about level with the 50-ml. mark on C, where it remains until the combustion is finished. Run from F into T 2 ml. of *combustion reagent* for a micro combustion of 2 to 3.5 mg. of carbon or 1.5 ml. for a sub-micro combustion of 0.3 to 0.7 mg. of carbon.

Heat with the micro flame. At first avoid heating so rapidly as to form a foam collar of more than 2 cm., although even if it fills the tube the error may be trivial; later heat rapidly to boiling. As carbon dioxide and oxygen are evolved, with consequent falling of the mercury in C and rising in the manometer, open cock a slightly every few seconds to admit mercury from the leveling bulb L into C, keeping the gas in C at about 2 ml. Within about a minute from the start of heating, when the gas has forced the mercury to the top of the manometer, open cock a completely and leave open to the end of combustion. Continue the vigorous boiling, with foam filling from one-third to one-half of the tube, at about 600 mm. pressure for 1.5 minutes. Even fatty acids are burned within that time, but with them vigorous boiling is particularly essential.

If desired to estimate the volume of oxygen evolved, after the carbon dioxide has been absorbed, but as the boiling continues, set the mercury meniscus in C at 50 ml., close a for a moment and note the height of the mercury in the manometer above the surface in C. If less than 350 mm., the subsequent 20 excursions will be sufficient.

Absorption of Carbon Dioxide. Continuing the boiling, lower the mercury in C to the 50-ml. mark and raise to the 5-ml. mark, repeating 20 times. If desired an aspirator can be intermittently applied to L to move the mercury up and down in C; this device saves manual labor. The transfer after 5, 10, 15, and 20 excursions was found to be 91, 98.3, 99.7, and 100% respectively. When the absorption is complete, remove the flame, close b and disconnect Q from C. Remove the hot combustion tube with tongs to an upright position in a weighted Pyrex Erlenmeyer flask (Fig. 31, B).

Ejection of Unabsorbed Gases. Before the oxygen and nitrogen are ejected, fill the curved inlet capillary above b with mercury drawn in from a small bottle by lowering L (Fig. 31, A). Then with b closed, raise L a little above b, close a, and open b to connect C with E. Admit mercury from L into C until the rising alkali solution, driving the gases out through E, just reaches the bottom of b. Close a, then b, lower L to the position shown in Figs. 30 and 31, and admit a little mercury from cup E into C to seal the connecting capillary. A small air bubble trapped in the capillary, thus readmitted into C, is not detrimental since it is carbon dioxide-free.

Extraction of Carbon Dioxide. Measure exactly 1 ml. of *2 N lactic acid* into C through the mercury seal from an accurate stopcock pipet with a rubber-ringed tip. After the acid, admit mercury to fill the capillary between E and C. Lower the mercury in C to the 50-ml. mark, close a, and shake for 20 or 30 seconds, thus extracting most of the car-

bon dioxide from the solution. To correct for the lowering of the mercury below the 50-ml. mark caused by the carbon dioxide that has entered the gas phase, admit mercury from L to bring the level exactly to the 50-ml. mark and shake for 1.5 minutes to complete the extraction of the carbon dioxide.

Admit mercury until the volume of the gas phase is reduced to 10 or 2 ml. for 2 to 3.5, or 0.4 to 0.7 mg. of carbon respectively, with precautions to avoid reabsorption of carbon dioxide given by Van Slyke and Neill [3] or by Peters and Van Slyke.[4] The chief precautions are to complete the admission of the mercury within 30 or 40 seconds and to avoid causing oscillations of the mercury in the chamber and manometer by jerky manipulation of cocks. Following these precautions, the reabsorption during the admission of the mercury is so constant (0.7 and 1.6% for compression of the gas to 10 and 2 ml. respectively) that the variation is no more than 1 part in 1000 and 300 parts respectively. Use a hand lens to bring the aqueous meniscus up *exactly* to the 10- or 2-ml. mark.

Manometer Reading of p_1. Take the reading; then for a duplicate reading (unnecessary after practice) again lower the mercury to the 50-ml. mark, shake for a minute, and return the gas to the 2- or 10-ml. mark and repeat the p_1 reading.

Reabsorption of Carbon Dioxide. Open the cock a with the bulb left at the level shown in the figures so that the gas is under slight negative pressure. Into cup E, measure 0.5 ml. of *1.0 N sodium hydroxide solution* by counting drops and admit the *alkali* into C, avoiding admission of air into either the chamber or capillary above b. In submicro combustion with less than 2 ml. of carbon dioxide, the *alkali* may be admitted during 10 seconds; with larger amounts extend the admission through a period of 30 seconds or more. If the alkali flows down in a solid column into the 4-mm. tube at the top of the chamber, instead of streaming down the walls, dislodge with about 1 ml. of mercury admitted in fine jets. When all the alkali, except enough to fill the capillary below E, is in C, pour 2 or 3 ml. of *acidified water* into the cup followed by 0.5 ml. of mercury which in turn is run into C, dislodging the alkali. To mix the solution in C and insure complete absorption of the carbon dioxide, lower the mercury three times to a little below the 10-ml. mark and raise each time to bring to atmospheric pressure.

Manometer Reading of p_2. Bring the solution meniscus in the chamber to a point a little below the 10- or 2-ml. mark at which p_1 was read and let drain for 1 minute. During this minute read the temperature in the water jacket and look up the calculation factor in the table (below). Finally raise the meniscus exactly to the 2- or 10-ml. mark and read p_2 on the manometer. Calculate the pressure (P_{CO_2}) of carbon dioxide from $P_{CO_2} = p_1 - p_2 - c$ in which c is the $p_1 - p_2$ of a blank analysis.

Washing Chamber. Eject the alkaline lactate solution from C and wash once with *dilute acid* and once with water thus: Lower L about 80 cm. below C so as to drain all the mercury from the chamber. During the draining place a few drops of *2 N lactic acid* in the cup above, then water to the top. Run the acidified water (but no air) into the evacuated chamber and expel by raising L. Repeat, using water only. After ejecting the water, lower the mercury again to the bottom of C and let rise slowly to dislodge any water and float it on the mercury. After running up the water thus collected, together with 1 ml. of mercury, into the cup, the apparatus is ready for the next combustion.

Alternate Technique without Reabsorption of Carbon Dioxide. The procedure saves 3 or 4 minutes but is slightly less exact. Proceed as above until p_1 has been read, then calculate P_{CO_2} as $P_{CO_2} = p_1 - p_0$ in which p_0 is

the p_1 reading of the blank at the same temperature. If the blank analysis was made at within 3° of the same temperature as the unknown, correct the blank reading to apply to the unknown by calculating p_0 as (p_1 of the blank) + (rise in p_{H_2O}) in which "rise in p_{H_2O}" is the increase in vapor tension of water caused by the difference in temperature of the actual and blank analysis. For example, if the blank is run at 20° where the vapor tension of water is 17.4 mm. and the analysis of the unknown is run at 22° where the vapor tension is 19.6 mm., the p_0 used is p_1 of the blank plus 2.2 mm. For reverse temperatures, the p_0 used would be p_1 of the blank minus 2.2 mm., the "rise in p_{H_2O}" being negative. Vapor tensions are given in the last column of the table below.

Between analyses without reabsorption, wash the chamber only once, with water.

Calculation of Micro and Submicro Analyses. Obtain the milligrams of carbon in the sample by calculation, using the factor found in the table (below), corrected if necessary by a factor obtained from control analyses of a standard pure substance as described in the next paragraph.

Correction of Factor by Combustion of Pure Substance. In order to include the corrections for volume of the large and small bulb of the manometer chamber, the thermometer, and the rider of the micro balance in one correction factor (b), determine the carbon in an easily analyzed substance of high purity (such as dextrose) and solve the following equation for both micro and sub-micro combustions:

$$\frac{C}{C^1}$$

in which C is the carbon present and C^1 is the carbon calculated from P_{CO_2} by the factor. Although the factors in the table may be used unchanged for ordinary work, using apparatus furnished by reputable manufacturers, it is desirable that each analyst prepare his own table of corrected factors. These may be plotted as a curve against temperature on a sufficiently large scale to permit an accuracy in reading of 1 part in 3000.

Analyses at Low Room Temperature. The method has been controlled by analyses made from temperatures above 18° and most of them at 22° or higher. At lower temperatures, it may be necessary to increase the excursions for absorption of carbon dioxide from 20 to 25 and the extraction from 1.5 to 2 minutes or, for temperatures below 15°, to 2.5 minutes.

Care of Apparatus. After a day's combustions, wash out cup F with *sirupy phosphoric acid*, remove cock, lubricate with *phosphoric acid*, and replace. If through neglect the cock freezes, it can usually be loosened by hot water. Cover tube Q and leave inserted in the combustion tube last used, which is placed upright in an Erlenmeyer flask weighted with lead shot. Rinse the combustion tubes once with tap water, then three times with distilled water and store inverted in a covered fruit jar. Never leave the tubes open in the circulating air of the laboratory, except when receiving the charge. Always clean new tubes by heating with *chromic acid* cleaning mixture to 120 to 140°, then rinse, and dry. If alkali from the chamber gets into Q, discard the analysis, rinse with *dilute hydrochloric acid*, followed by water, and dry.

B. Macro Combustion. Measure the carbon dioxide pressure when the mercury in C is at the 50-ml. mark, that is, with 46 ml. of gas, allowing 4 ml. for the solution. Carry out the details as directed under Alternate Technique with Reabsorption above. Five analyses may be made in 1 hour.

Apparatus. Except that T is a 25-ml. instead of a 15-ml. tube and F holds 5 instead of 2 ml., the *assemblage* is the same as given under A.

Reagents. *Combustion Reagent.* Prepare as under A, except that 30 instead of 25 g. of chromium trioxide is used.

Alkali-Hydrazine Solution. Weigh 6 g. of hydrazine sulfate into a 100-ml. volumetric flask and fill to the mark with carbon dioxide-free 3 N sodium hydroxide solution. Other details as under A.

Lactic Acid, 5 N. Dilute concentrated lactic acid (sp.gr. 1.20) to 2 volumes with water and adjust to 5 N after titrating a fifty-fold dilution against 0.1 N sodium hydroxide solution (phenolphthalein).

PROCESS. Weigh a charge containing 8 to 15 mg. of carbon, add 300, instead of 200, ml. of *potassium iodate solution*, and pour into F enough *combustion reagent* to deliver 5 ml. Eject the air three times. Measure 2 ml. of *alkali-hydrazine solution* as under A. Conduct the combustion, absorption of carbon dioxide, and ejection of unabsorbed gases likewise as under A, except that 5 ml. of *combustion reagent* are used and the boiling after the pressure reaches 600 mm. is for 3 instead of 1.5 minutes to compensate for the greater ratio of carbon to chromic acid.

Set free the carbon dioxide with 2 ml. of *5 N lactic acid* measured into C from a stopcock pipet. Extract the carbon dioxide. During the last 1.5 minutes of the extraction have the mercury meniscus exactly at the 50-ml. mark. Read p_1 with the mercury meniscus at the 50-ml. mark. The carbon dioxide is not reabsorbed with alkali; too much heat would be generated. The "Alternate Technique without Reabsorption of Carbon Dioxide," described above, is used, with p_0 determined as p_1 in a blank analysis.

CALCULATION. In calculating the pressure of carbon dioxide use the formula

$$P_{CO_2} = p_1 - p_0$$

Calculate the milligrams of carbon in the sample by multiplying P_{CO_2} by the factor from the table. Test the factors in the trial combustion of dextrose or other pure substance as directed under A and correct if necessary by the b factor found.

FACTORS FOR CARBON CALCULATION
(VAN SLYKE AND FOLCH)

Temperature	Factors *			Vapor Tension of Water
	Submicro analysis $a = 2.0$ ml.	Micro analysis $a = 10.0$ ml.	Macro analysis $a = 46$ ml.	
°C.				mm.
10	0.001474	0.007303	0.03320	9.1
11	466	265	304	9.8
12	458	228	289	10.4
13	451	192	274	11.1
14	444	157	269	11.9
15	437	122	244	12.7
16	430	088	229	13.5
17	424	054	215	14.4
18	417	020	201	15.3
19	410	0.006987	187	16.3
20	403	954	173	17.4
21	397	922	159	18.5
22	390	890	145	19.6
23	384	859	132	20.9
24	378	828	119	22.2
25	372	798	106	23.5
26	366	769	093	25.0
27	360	740	080	26.5
28	354	711	067	28.1
29	349	683	055	29.7
30	343	655	043	31.5
31	337	628	031	33.4
32	332	601	019	35.3
33	327	575	007	37.4
34	321	549	0.02996	39.5
35	316	523	985	41.8

* The values for a are the gas volumes when the pressures are read.

c. NITROGEN

The two following methods, before the introduction of the Kjeldahl method, were the only dependable means of determining nitrogen in food products and still are described in all works on general and organic analysis.

Dumas Copper Oxide Dry Combustion Method. The Johnson and Jenkins modification[5, 6] of this standard method, commonly

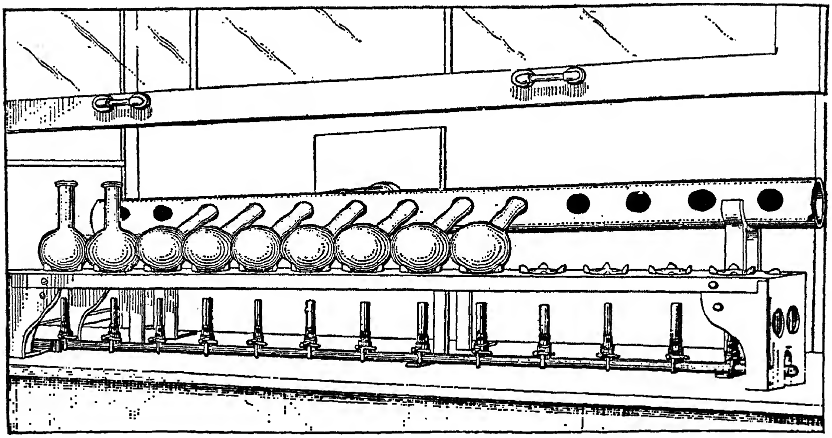


FIG. 32. Johnson Multiple Kjeldahl Digestion Stand.

known as the absolute method, employs an improved Schiff azotometer.[7] It may be regarded as the court of last resort, since it is believed to be suited, sometimes with modification, for all nitrogenous substances, natural and synthetic. The nitrogen is measured as such and the calculation takes into account temperature and barometric pressure.

Varrentrapp and Will Soda-Lime Dry Combustion Method. For ordinary combustion in the absence of nitrates, nitrites, and some other difficultly oxidized constituents, this method was standard. The nitrogen of proteins burns to ammonia and is titrated as in the Kjeldahl method.

Kjeldahl Wet Combustion Method. As first proposed by Kjeldahl,[8,9] the digestion was carried out with a mixture of *sulfuric acid, fuming sulfuric acid,* and *phosphoric anhydride,* and the oxidation was completed by the addition of *potassium permanganate.*

The four modifications of the Kjeldahl method that have withstood the test of time, employing as their distinctive reagents *mercuric oxide, potassium sulfate, copper sulfate,* and *phenol,* are those of Willfarth, Gunning, Arnold, and Jodlbauer, respectively. The Arnold modification is applicable in the presence of alkaloids and the Jodlbauer modification in the presence of nitrates.

I. Willfarth Mercuric Oxide Macro Modification.[10] APPARATUS. *Digestion Stand.* Where only an occasional determination is made, the digestion may be carried out on a triangle resting on a lamp stand over a free flame, the neck of the flask being supported on a second ring turned to one side to incline the flask at the proper angle. An electric unit has the advantage that the digestion may be carried out in the open air on a window sill. The multiple Johnson digestion stand, shown in Fig. 32, is cast from iron after Winton's pattern. Each hole is provided with three projections that hold the flask when on a slant during digestion and three uprights that act as guards when the glass ware is in an upright position. The Boltwood fume conductor consists of a 4-in. lead pipe with holes into which the flasks project.

Distillation Apparatus. The apparatus designed by Polenske (Fig. 108) for distillation of volatile fatty acids is well adapted for a single nitrogen determination, if a receiver

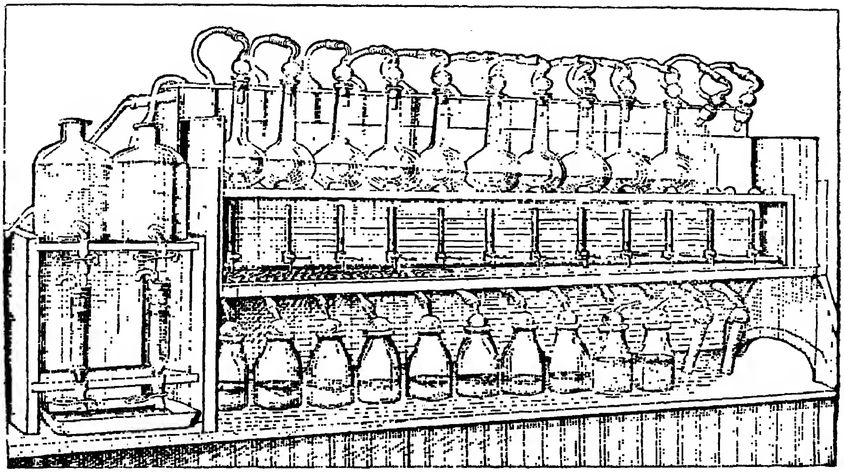


FIG. 33. Johnson Multiple Kjeldahl Distillation Apparatus.

and bulb tube are substituted for the double graduated flask. The Johnson multiple distillation apparatus with accessories (spray traps, suspended reagent tubes, and milk bottles for receivers) added by Winton is shown in Fig. 33. The pint milk bottles are readily grasped below the ring-neck in titrating.

Combination Apparatus. Many forms of multiple digestion and distillation apparatus, for both gas and electric heating, are now supplied by apparatus houses. An all electric combined digestion and distillation assembly, made for the New Mexico State College by the Precision Scientific Co., Chicago, is shown in Fig. 34.

Burets. Ordinary burets (Fig. 35) are of the proper caliber for 0.1 N acid and alkali; these are recommended for an occasional analysis, particularly in a student laboratory. The ball cock permits splitting of the drops. The Squibb buret attached to a 5-pint reagent bottle is a great convenience (Fig. 36).

For routine work on a large scale (50 to 75 determinations per person per day), special burets of narrow bore (1 ml. = 2.6 cm.) permit accurate titration with standard solutions of such a strength that 1 ml. of the acid is equivalent to 1% of nitrogen (when working on a gram of material). The delicacy of the back titration is increased by using an alkali solution 2.5 times weaker than the acid solution in a specially graduated buret with a reading of 1 representing 2.5 ml., thus exactly corresponding in neutralizing power with a reading of 1 on the acid buret. The calculation is simply one of subtraction.

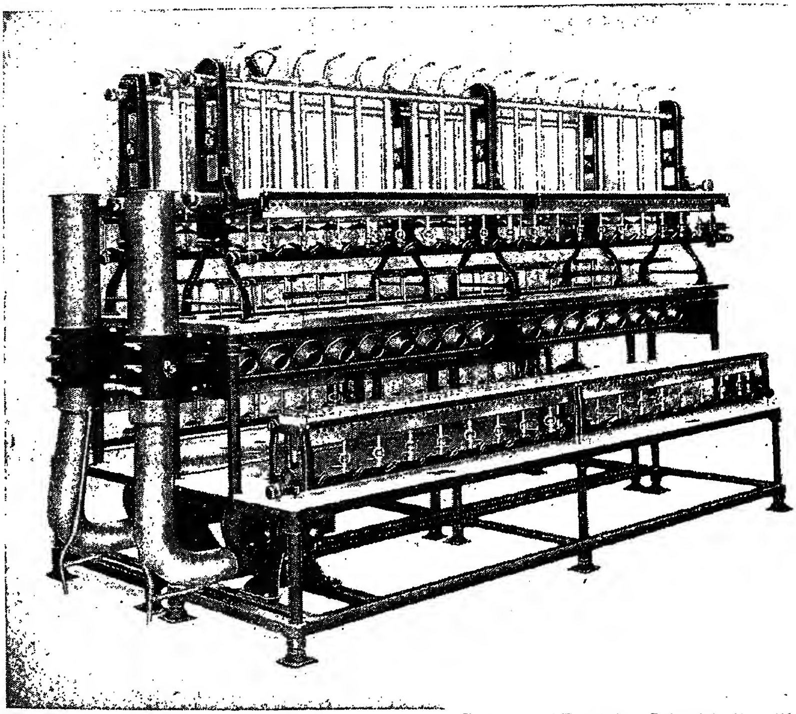
REAGENTS. *Sulfuric Acid.* Commercial oil of vitriol usually contains less nitrogen, as shown by blank determination, than the so-called c.p. acid.

Mercuric Oxide (mercury oxide red). The crystalline powder.

Potassium Permanganate. The c.p. crystals.

Potassium Sulfide Solution, 4%.

Sodium Hydroxide Solution, nearly saturated. Empty one 10-lb. can of granular caustic soda (Greenbank) into an iron pot, add 4350 ml. of water, and stir constantly with an iron rod until dissolved. Cover, allow to stand overnight, decant off the clear liquid, and store in rubber-stoppered bottles.



Courtesy of Precision Scientific Co., Chicago

FIG. 34. Precision Multiple Kjeldahl Digestion and Distillation Assembly.

Indicators. Cochineal tincture: Digest 24 g. of powdered cochineal bugs in a mixture of 400 ml. of ethanol and 1 liter of water. Let stand overnight and filter. *Methyl red* (0.2 g. in 100 ml. of ethanol), *Congo red* (0.5 g. in 90 ml. of water and 10 ml. of ethanol), and *methyl orange* (0.1 g. in 100 ml. of water) are also satisfactory, provided the same indicator is used in standardizing as in the actual determination. Do not use phenolphthalein.

Zinc, granulated, 20 mesh.

Standard Ammonium Hydroxide Solution, 0.1 *N*. Dilute 28 ml. of NH_4OH (28%) with 2 liters of water. Adjust to correspond with the 0.1 *N* acid.

Ammonium hydroxide is preferred to potassium or sodium hydroxide because it is not caustic and does not cement the stopcock, etch the buret, or cloud the solution. There is, however, a gradual loss of ammonia if not kept well stoppered and in a cool place. Before measuring this, as well as all volumetric solutions, a few drops should be drawn down and rejected to clear the delivery tube.

Standard Hydrochloric Acid, 0.1 *N*. Dilute 17 ml. of HCl with 2 liters of water. Standardize by one of the methods given below, then by proportion calculate the amount of water or acid required to make the solution exactly 0.1 *N*. Finally standardize the adjusted solution by two methods.

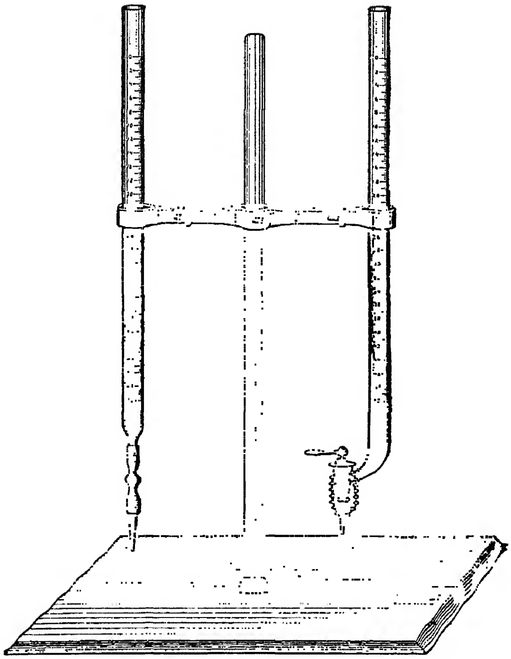


FIG. 35. Burets with Ball and Glass Stopcocks.

Hydrochloric acid is preferred to sulfuric acid because standardization as silver chloride is more accurate than that as barium sulfate; furthermore, being volatile, it causes less damage.

Of the three methods of standardization that follow obviously only (*b*) and (*c*) are applicable to standard acids other than hydrochloric.

(*a*) *Standardization with Silver Nitrate.* Carefully measure into a beaker from the buret to be used in the actual analysis 100 ml. of the solution, add $AgNO_3$ solution in slight excess and a few drops of HNO_3. Heat nearly to boiling with constant stirring until the AgCl precipitate flocks and the supernatant liquid is clear. Filter on a porcelain Gooch crucible, wash three times with hot water by decantation, stirring after each addition, and finally on the crucible. Dry cautiously on a hot plate and heat below redness until the silver chloride contracts to a mass and finally fuses at the edges. Multiply the weight of silver chloride (AgCl) by 0.0009765, thus obtaining the grams of nitrogen equivalent to 1 ml.

(*b*) *Standardization with Sodium Bicarbonate.* Anhydrous sodium bicarbonate of proper purity is readily obtainable. Because

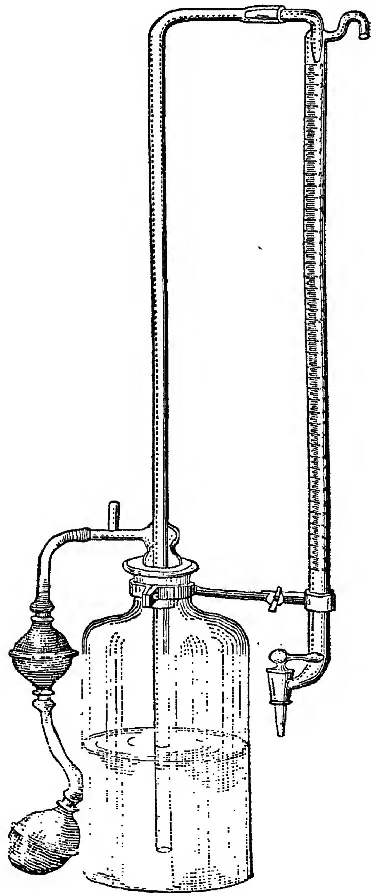


FIG. 36. Squibb Buret with Filling Device.

of its stability it is preferable to the normal carbonate. The remainder of a pound, acquired forty years since, from the Royal Baking Powder Co. (now Royal Manufacturing Branch of Standard Brands Inc.), is still of theoretical composition.

Weigh about 0.7 g. of $NaHCO_3$, free from the Na_2CO_3, into a beaker, add 15 ml. of water and 100 ml. of standard HCl, which must be in slight excess. Cover with a watch-glass and evaporate the solution to at least half its volume by gentle boiling. Cool and titrate back with standard NH_4OH, using cochineal or other suitable indicator. Multiply the weight of $NaHCO_3$ by 0.1667 and divide by the corrected milliliters of standard acid, thus obtaining the weight of nitrogen equivalent to 1 ml. of the standard acid.

If anhydrous Na_2CO_3 is preferred, as described by Fresenius ignite at dull redness about 0.8 g. of the carbonate in a platinum crucible, cool in a desiccator, and weigh. Dissolve in 15 ml. of water, add 50 ml. of standard HCl, and proceed as above. The factor is 0.26428.

(c) *Standardization with Borax.* Standard Borax ($Na_2B_4O_7 \cdot 10H_2O$).[11] Saturate 300 ml. of water with $Na_2B_4O_7 \cdot 10H_2O$ at 55° or below, filter, cool to 10°, and allow to crystallize with continual agitation. Decant the mother liquor, wash the crystals with 25 ml. of cold water, dissolve in about 200 ml. of water at 55°, then recrystallize and wash as before. Collect on a Büchner funnel and wash with 25 ml. of ice cold water, followed by two 20-ml. portions of ethanol and two 20-ml. portions of ether. Dry the crystals at room temperature or over a solution saturated with both sugar and salt. Allow to stand 24 hours and store in a glass-stoppered bottle in a desiccator.

Process. Weigh into a 300-ml. Erlenmeyer flask standard borax equivalent to about 40 ml. of the standard acid. Dissolve in 40 ml. of CO_2-free water, stopper, swirl until dissolved, add 4 ml. of methyl red indicator (0.1% in 57% ethanol), and titrate to a color matching that of the reference solution, that is, 0.1 M in boric acid and 0.05 M in sodium chloride.

Calculation. Obtain the normality (N) by the formula

$$N = \frac{B}{A \times 0.19072}$$

in which B is the grams of $Na_2B_4O_7 \cdot 10H_2O$ and A is the milliliters of standard acid.

PROCESS. *Digestion.* Weigh into a 500- to 600-ml. flat-bottom flask of Pyrex or Jena glass a quantity of the sample equivalent to about 1 g. of dry matter. Add about 0.7 g. of powdered *mercuric oxide* (measured conveniently from a 22 pistol cartridge shell cut to proper length and soldered to a wire handle) and 20 ml. of *sulfuric acid* free from nitrogen. If at a later stage the acid seems insufficient for safe boiling, add 5 ml. more or repeat with the larger amount.

Heat for a time at a low temperature, then bring to boiling, continuing the heating for a time after the liquid is colorless or light yellow. During the boiling the flask should be turned and gently shaken to rinse down the carbonaceous matter from the sides of the flask. With the flask in an upright position, add slowly, while shaking, *potassium permanganate crystals* until the color persists. This addition of permanganate was regarded by Willfarth as superfluous and is omitted by some analysts.

Distillation. After cooling, dilute with 200 to 250 ml. of water, add 25 ml. of 4% *potassium sulfide solution,* and, while rotating the flask, *nearly saturated sodium hydroxide solution* until a piece of test paper shows an alkaline reaction. Then, without delay, add a pinch of finely granulated *zinc* and connect with the distilling apparatus, a measured excess of 0.1 N *acid* having been added previously to the receiver and diluted with sufficient water to trap the bulb tube.

Heat the distilling flask with constant watching until the solution boils without danger of frothing over, continuing the boiling until 150 to 200 ml. of liquid have passed over. Bumping of the liquid is a sure sign that the boiling must be stopped at once. Disconnect the receiver without delay and wash off the bulb of the delivery tube. It is well to replace the receiver by another containing water which, by sucking back into the cooling flask, cleans the tube. This is imperative if any foam has passed over. Addition of a bit of *paraffin* to the distilling flask is a last resort, if foaming is persistent.

Titration. Titrate back with *standard alkali.* The indicator should be the same as used in the standardization.

CALCULATION. Subtract from the volume of standard acid used the volume of standard alkali required for the titration and multiply by the weight of nitrogen corresponding to each milliliter of the standard acid, then multiply by 100 and divide by the weight of the charge, thus obtaining the per cent of nitrogen.

II. Gunning Potassium Bisulfate Macro Modification.[12] In this modification, the boiling point is raised by the addition of potassium sulfate to the sulfuric acid, thus forming potassium bisulfate, which takes the place of mercuric oxide or other special metallic oxidizing agent. It has the advantage of fewer reagents and a colorless solution in which phenolphthalein shows the alkaline reaction. Care must, however, be taken to secure complete oxidation; otherwise the results may be low.

Gunning in his original description directed that 18 g. of potassium sulfate be used and in the writers' experience this amount or at least 15 g. is essential for obtaining the full amount of nitrogen in certain substances. Some chemists, however, recommend smaller amounts which facilitate boiling and the rinsing down of carbonaceous matter from the sides of the flask.

REAGENTS. These are *sulfuric acid, commercial potassium sulfate, phenolphthalein indicator*, and *nearly saturated sodium hydroxide solution.*

PROCESS. Digest a quantity of the material, not exceeding 1 g. of dry matter, with 20 ml. of *sulfuric acid* and 15 g. of *potassium sulfate* in a 550- to 600-ml. Pyrex or Jena flask as in the Willfarth modification, but omit the addition of potassium permanganate. If necessary, add a little more *sulfuric acid* during the later stages.

Cool, dilute with about 225 ml. of tap water, add a few drops of *phenolphthalein indicator*, then, with continual rotation, *sodium hydroxide solution* to alkaline reaction, and a pinch of 20-mesh granulated *zinc*, finally connecting with the distillation apparatus. Proceed with the distillation and titration as in the Willfarth modification.

III. Arnold Copper Sulfate Macro Modification. Arnold and Wedemeyer[13] introduced blue vitriol as a new digestion reagent which, in conjunction with mercuric oxide and potassium sulfate, brings about a complete conversion to ammonia of the nitrogen in certain alkaloidal products. In the writers' experience, the Arnold modification gives the true amount of nitrogen in black and white pepper (Part II, J1) containing the alkaloid piperine.

PROCESS. Follow the general instructions given above for the Willfarth modification, but in addition to 1 g. of *mercuric oxide*, use for the oxidation 16 g. of *potassium sulfate* and 1 g. of *copper sulfate*, followed by 25 ml. of *sulfuric acid.* The *potassium sulfide solution* added later should be sufficient to precipitate the copper as well as the mercury.

IV. Lauro Selenium Macro Modification.[14] By the substitution of 0.1 to 2 g. of *selenium* or *selenium oxychloride* for the oxidizing agents of the foregoing modifications, Lauro, a New York consulting chemist, shortens materially the time of digestion. Using 25 ml. of *acid*, 10 g. of either *sodium* or *potas-*

sium sulfate together with *mercuric oxide*, *copper sulfate*, or *selenium*, the average time required for the digestion was respectively as follows: flour 78, 62, or 17; wheat 118, 97, or 22; cottonseed meal 127, 105, or 37; and cracklings 162, 137, or 47 minutes.

NOTES. Tennant, Harrell, and Stull,[15] of the New York Hospital, report a similar shortening of the time of digestion by using *selenium* in place of copper sulfate and West and Brandon in the micro method (below) use *selenium oxychloride* in preference to hydrogen peroxide.

Rice[16] states that addition of 0.2 ml. of *selenium oxychloride* to the digestion mixture in combination with *copper sulfate* shortens the digestion at least a half hour.

Sandstedt,[17] using 0.1 g. of *copper*, 0.7 g. of *mercuric oxide*, and 0.1 g. of *selenium*, found that the digestion time for a high protein flour was 1 hour, 45 minutes, and 45 minutes and the cost of the catalyst 0.002, 0.48, and 0.15 cents respectively. He notes that selenium has the advantage of not requiring a precipitant, but warns against the greater danger of loss of nitrogen by overdigestion.

Messman[18] prepares a mixture of 90 parts of *sodium sulfate*, 7 parts of *mercuric sulfate*, 1.5 parts of *copper sulfate*, and 1.5 parts of *powdered selenium*. Eight grams of this mixture are used in conjunction with 20 ml. of acid.

Taylor,[19] of the Missouri Department of Agriculture, prepares a flux of 100 parts of *sodium sulfate* ($Na_2SO_4 \cdot 7H_2O$), 7 parts of *mercuric oxide*, and 1.5 parts of *powdered selenium*. Ten grams of this flux are added to the sample, together with the normal amount of *sulfuric acid*.

In the Hengar micro technique,[20] a single pedestal stand (Fig. 37) supports the 100-ml. flask during both digestion (without fume conductor) and distillation (without water cooling). A charge of 0.05 to 0.1 g., weighed into a paper cup, is digested with 2.5 ml. of *sulfuric acid*, 1 to 1.5 g. of *potassium sulfate*, and a *selenized granule* containing an anti-bump that remains after the selenium has dissolved. A tube wrapped with filter paper prevents contamination of the air with sulfuric acid mist. For the distillation, 35 ml. of water and 4 to 5 g. of *sodium hydroxide pellets* are added.

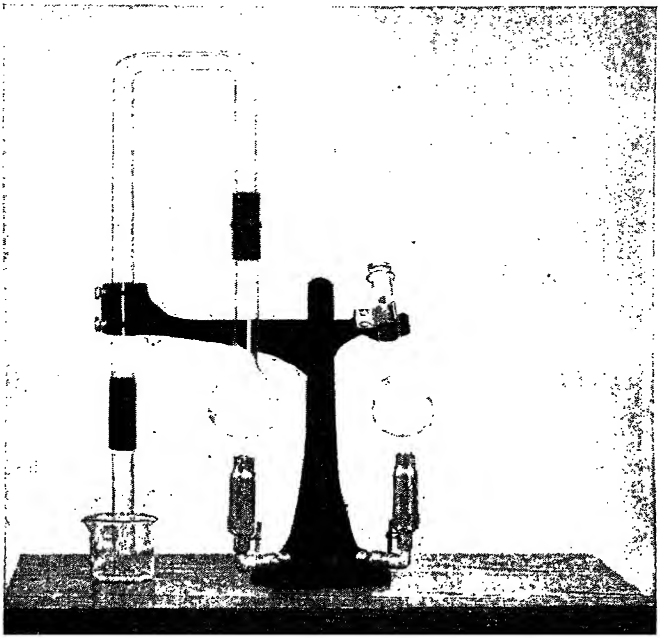
Hitchcock and Belden,[21] of the Yale Medical School, describe a semi-micro procedure employing *selenium oxychloride* and *mercuric oxide*, with due credit for certain details to Northrop,[22] Oxborne and Krasnitz,[23] and Taylor.[24]

Five milliliters of the solution, containing 5 to 6 mg. of nitrogen, are digested with 2 ml. of *sulfuric acid*, 1 g. of *potassium sulfate*, 70 mg. of *mercuric oxide*, and 2 drops of *selenium oxychloride*, bits of *alundum* being added as an anti-bump. The water passes off in 20 minutes and the mixture clears in 6 to 8, then the heating is continued 20 minutes longer. After addition of 35 ml. of water and 5 ml. of *50% sodium hydroxide solution* containing about 8% of *anhydrous sodium thiosulfate*, the distillation and titration are carried out in the normal manner, using *methyl red* as indicator.

V. Jodlbauer Phenol Macro Modification.[25] This method serves for the determination of total nitrogen in materials containing nitrates. Nitrates form with the phenol nitrobenzene, which is reduced by zinc dust to an amido compound related to, if not identical with, aniline, which in turn is converted by digestion into ammonium sul-

REAGENT. *Phenol-Sulfuric Acid.* Dissolve 40 g. of phenol in 1 liter of H_2SO_4.

PROCESS. *Formation of Nitrobenzene.* Weigh 1 g. of the material into a Kjeldahl flask and add in two portions 30 ml. of *phenol-sulfuric acid.* After adding the first portion, shake thoroughly with a rotatory motion to prevent caking; then use the second portion to rinse down the sides of the flask. Shake frequently with a gentle rotatory motion dur-



Courtesy of Hengar Company, Philadelphia

FIG. 37. Hengar Micro-Kjeldahl Apparatus.

ing 0.5 hour and occasionally for another 0.5 hour, taking care that the material is not distributed over the sides of the flask.

Reduction. Add 2 g. of *zinc dust* slowly with shaking, holding the flask perpendicular to avoid contact with the neck.

Oxidation. Heat at first over a low flame, then raise the heat gradually and boil until the liquid is of a dull red color. Add 0.7 g. of *mercuric oxide* and boil until the solution is of a light straw color, but no longer. Add *potassium permanganate* and proceed as in the Willfarth modification.

NOTES. Scovell[26] substituted for the phenol-sulfuric acid a solution of 60 g. of *salicylic acid* in 1 liter of *sulfuric acid* without loss in accuracy.

Winton[27] further modified the method by substituting for the mercuric oxide 10 g. of *potassium sulfate*, such as is employed in the Gunning process, omitting the addition of permanganate and sulfide. In this process, the larger quantity of potassium sulfate, employed in the regular Gunning process, is not only unnecessary but detrimental.

VI. Olsen Reduced Iron Macro Modification.[28] Pucher *et al.*,[29] confirming the findings of Ranker,[30] found that the Scovell salicylic acid-zinc method does not give the full amount of nitrogen in tobacco and other plant materials containing nitrates. They credit Olsen with priority in the determination of total nitrogen by reduction of nitrates with iron (Ulsch Method, Part I, C4*f*) and wet combustion.

Pucher, Leavenworth, and Vickery Technique.[29] PROCESS. *Reduction.* Pipet a water extract of the sample into a Kjeldahl flask,

dilute to 30 to 40 ml., then add 10 ml. of *1 + 1 sulfuric acid* and 3 g. of *reduced iron powder*, shake for 10 minutes at room temperature, slowly heat to boiling, and boil for 5 minutes.

Digestion. Cool, add 30 ml. of *sulfuric acid*, a drop of *mercury*, and 5 g. of *anhydrous sodium sulfate.* Remove the water by cautious heating and digest at a higher heat until the liquid is clear and the precipitate assumes a yellow color. Continue the heating for 1 to 2 hours longer. While it is hot, drop in a few crystals of *potassium permanganate.*

Distillation. Cool, dilute with 300 ml. of water, add 3 to 5 g. of *sodium thiosulfate*, a small piece of *paraffin*, an excess of *sodium hydroxide solution*, and a little *zinc*, then distil and titrate in the usual manner.

VII. Koch and McMeekin Hydrogen Peroxide Micro Modification.[31] The procedure, as employed by its originators at the University of Chicago, gave results on milk, urine, and blood, and also on pure substances, which agreed well with those by the Kjeldahl-Gunning method. When applied to miscellaneous foods, however, a special technique may be necessary. It is shorter than the Folin and Denis procedure and also avoids the separation of silicon dioxide.

REAGENTS. *Hydrogen Peroxide Solution, 30%.* Merck's *Superoxol* or Kahlbaum's *perhydrol.*

Modified Nessler-Folin Reagent.[32] To a solution of 22.5 g. of iodine in 20 ml. of water containing 30 g. of KI add 30 g. of pure metallic mercury, shake well while cooling under the tap until the supernatant liquid has lost all its color due to iodine, decant the supernatant liquid, and test by adding a few drops to 1 ml. of 1% starch solution, which must be positive. To the remaining solution add a few drops of iodine solution, of the same concentration as employed above, until there is a slight excess of free iodine as shown by adding a few drops to 1 ml. of starch solution, then dilute to 200 ml. and mix well. Add this solution of potassium mercuric iodide to 975 ml. of an exactly 10% NaOH solution, mix well, and allow to settle.

Stock and Standard Ammonium Sulfate Solutions, in 0.05 *N* sulfuric acid. Prepare as directed by Folin and Denis[32] as follows: Dry c. p. $(NH_4)_2SO_4$ for 1 hour at 110° or over H_2SO_4 for a day or two. Dissolve 4.716 g. in 1 liter of 0.2 *N* H_2SO_4. Dilute this stock solution (1 mg. of nitrogen per 1 ml.) to a concentration of 1 mg. per 20 ml.

PROCESS. *Digestion.* Pipet into a 200 x 25 mm. Pyrex test tube an aliquot of a sample of milk or other liquid, containing 0.3 to 1.0 mg. of nitrogen, add 1 ml. of *1 + 1 sulfuric acid*, and heat over the free flame, while shaking, or on the sand bath, until the water has been driven off, then heat further over the micro-burner until dense white fumes appear. Cool for 15 to 30 seconds, add 1 to 5 drops of *30% hydrogen peroxide* and continue the heating until white fumes again appear. If a discoloration appears, add several drops of the *peroxide.* In any event continue the heating for 2 to 5 minutes, then, when the digestion is complete, transfer the solution to a 100-ml. volumetric flask and dilute to 75 ml.

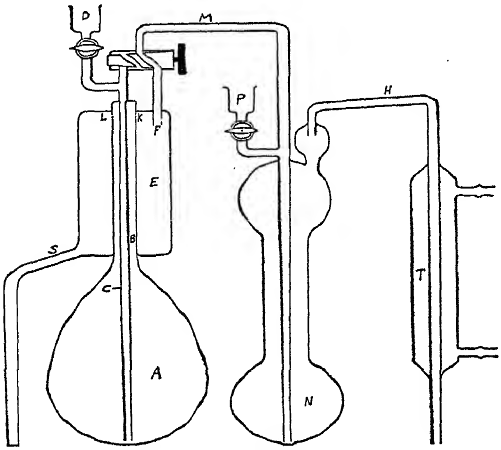
Color Formation. Cool the solution, add 15 ml. of *modified Nessler-Folin reagent*, dilute to 100 ml., and mix well.

Color Comparison. After 5 to 20 minutes, compare with a *standard* prepared by mixing in a 100-ml. volumetric flask 1.5 to 5 ml. of *standard ammonium sulfate solution* (containing 0.3 to 1 g. of nitrogen) with 1 ml. of *1 + 1 sulfuric acid*, diluting to about 75 ml., adding 15 ml. of *modified Nessler-Folin reagent*, and finally diluting to the mark. Correct for any nitrogen obtained in a blank determination.

VIII. Kemmerer and Hallett Modification.[33] The modification involves only the details of distillation as carried out in the determination of nitrogen in lake water.

APPARATUS (Fig. 38). *All Glass Distillation Assembly.*

PROCESS. *Distillation.* Add *ammonia-free water* through D to the steam generator A and run a blank; then add the digested sample with an excess of *potassium hydroxide* (indicated by a trace of copper sulfate previously added to the sample) through P to N.



Courtesy of Ind. Eng. Chem. 1937, **19**, 1296

FIG. 38. Kemmerer and Hallett Micro Kjeldahl Distillation Apparatus.

Conduct the distillation with the stopcocks as shown, with water running in T and small burners under A and N. The steam generated in A passes to N where it helps liberate the ammonia through H and on into T where it is condensed and collected in a 50-ml. graduated flask containing 25 ml. of *ammonia-free water.*

To clean the apparatus, allow the liquid in N to be drawn into E by the vacuum formed by the condensation of the steam. Rinse the distillation flask by placing a burner under A and turning the three-way cock to connect C and M, then when sufficient water has siphoned into N turn the stopcock, as in the cut, remove the flame and allow the rinse water to suck back into E.

Nesslerization. Dilute an aliquot of the distillate (usually one-half) to 50 ml. in a Nessler tube. Nesslerize and compare with a standard treated in like manner. If the amount of nitrogen is large, collect in *standard acid* and back-titrate.

NOTE. Bradley [34] (Newark College of Engineering), by distilling Kjeldahl digests into pure water only in a closed still, equipped with a safety balloon to relieve the pressure, and titrating the distillate directly with *standard acid,* reports an average recovery of 99.8% of ammonia. In an open still, an average of only 98.7% was recovered.

IX. Robinson and Shellenberger Persulfate Micro Modification.[35] The single reference to the literature on the previous use of potassium persulfate given by the authors (University of Washington) is erroneous. The results on cereal products agree with those by the Kjeldahl-Gunning method.

PROCESS. Char 10 to 20 mg. of the sample in a digestion tube with a small crystal of *copper sulfate* and 2 ml. of *sulfuric acid* (this requires about 4 minutes), then cool to at least 100°, add 1 g. of *potassium persulfate,* and heat gently until the persulfate is completely decomposed. A clear solution is usually obtained after heating 1 minute, and the digestion is completed in 10 minutes or less. The distillation apparatus is similar to that of Kemmerer and Hallett above.

X. West and Brandon Selenium Nesslerization Micro Modification.[36] In addition to being satisfactory for biological non-food material, the method was found by the originators (Washington University, St. Louis) to give the full amount of nitrogen when applied to creatine and creatinine in conjunction with 2 mg. of *sucrose.*

APPARATUS. *Pyrex digestion tubes,* 200 x 25 mm., constricted at the 25-ml. calibration mark.

REAGENTS. *Selenium Digestion Mixture.* To a mixture of 250 ml. each of H_2SO_4 and saturated K_2SO_4 solution, add 1 ml. of *sele-*

nium oxychloride. Decant the clear supernatant liquid after standing overnight.

Nessler Reagent.[37] Dilute with an equal volume of 10% sodium hydroxide solution. *Merck's Index* gives first place to the following: Dissolve 10 g. of $Hg(IO_3)_2$ and 5 g. of KI in 50 ml. of water and add 20 g. of KOH dissolved in 50 ml. of water.

PROCESS. Pipet a quantity of the solution, containing 0.2 to 0.3 mg. of nitrogen, into a Pyrex digestion tube and add 1 ml. of the *selenium digestion mixture* and 2 dry glass beads. Cover with a glass bulb and heat over a micro burner until all water is driven off, the material chars, and sulfate fumes appear. Reduce the flame and boil gently for 1 to 2 minutes until the solution becomes clear, then continue the boiling for 4 minutes.

Color Formation. Cool, dilute with 25 ml. of water, add quickly 15 ml. of *Nessler reagent,* dilute to the mark, close with a rubber stopper, and mix.

Color Comparison. Use for the comparison a standard prepared with 1 ml. of the *digestion mixture,* containing 0.2 to 0.3 mg. of nitrogen, treated with *Nessler reagent.*

XI. Winkler Boric Acid Modification.[38] Although classed as a modification of the Kjeldahl method, the procedure is in reality a distillation method for the determination of ammonia whether in an ammonium salt or the digest obtained by any wet combustion method.

As listed by Scales and Harrison [39] (U. S. Bureau of Plant Industry), boric acid as used in the Winkler method has four marked advantages: (1) it eliminates errors in measuring standard acid into the receiver, (2) the boric acid solution need not be accurately measured, (3) by using a suitable weight of sample, the percentage of nitrogen can be read directly from the buret, and (4) only one solution need be standardized. The authors named express a preference for *bromophenol blue* as the indicator.

A. Stover and Sandin Technique.[40] These authors (University of Alberta) have adapted the Winkler procedure to the micro determination of nitrogen, using a *mixed indicator* prepared from equal parts of *methyl red solution* (0.1% in ethanol) and *tetrabromophenol blue solution* (0.1 g. in 20 ml. of warm ethanol, diluted to 100 ml.). They state that the distillate does not need to be boiled before titration. The end-point is sharp. The color changes from a clear green in alkaline solution through a gray color at near the end-point to a final pale gray-violet with the slight amount of acid needed at the true end-point.

B. Wagner Technique.[41] The author (University of Pennsylvania), supplementing a paper by Meeker and Wagner,[42] gives the following specifications for macro, semi-micro, and micro determinations respectively: Capacity of Kjeldahl flask, 500, 100, and 15 ml.; nitrogen, normal limits 15 to 90, 1.5 to 7, and 0.4 to 1.4 mg.; sulfuric acid, most compounds, 20 to 30, 3 to 5, and 1 ml.; potassium sulfate (or 0.8 as much Na_2SO_4), 10, 1 to 2, and 0.4 g.; selenium, 0.2, 0.05, and 0.02 g.; mercuric oxide, 0.5, 0.1, and 0.03 g.; normal time of digestion after clearing, 60, 25, and 15 minutes; water added, 150 to 200, 35 to 50, and 6 ml.; volume distilled, 150, 25, 10 ml.

Capacity of Erlenmeyer flasks, 500, 100 or 150, and 50 or 100 ml.; boric acid-methyl red solution, 50, 25, and 5 ml.; carbon dioxide-free water, 150, 25, and 10 ml.; ordinary distilled water, 50, 25, and 10 ml.

Capacity and graduation of buret, 50/0.1, 25/0.1, and 10/0.05 ml.; normality of acid, 0.1 to 0.2, 0.02, and 0.01 *N;* limits of titration, 10 to 50, 5 to 25, and 3 to 10 ml.; and volume at end-point, 210 to 250, 55 to 75, and 18 to 25 ml. Titrate on a white surface with standard acid until a pink tint appears, but before it is as intense as the control.

C. Taylor and Smith Potentiometric Technique.[43] A study was made (University of Illinois) of two procedures with the distillate

collected in boric acid solution, namely, (1) titration with standard acid to a definite pH and (2) determination of the amount of ammonia absorbed by the boric acid by the change in pH. The former was found to be somewhat more accurate, but the latter (here described) was deemed better suited for routine work.

Apparatus. *Pregl Micro Distillation Assembly.*[44]

Beckman pH Meter, industrial direct-reading model.

Reagents. *Boric Acid*, 4%. Standardize and plot a curve for each lot as follows: Add to 10-ml. portions of the 4% H_3BO_3 solution, measured exactly in a micro pipet and diluted to 150 ml., definite amounts of *0.01 N NH_4OH solution.* Determine in each the pH and the ammonia by titration with 0.01 N H_2SO_4 to the pH represented by the original boric acid solution diluted to 150 ml. Prepare a calibration curve with pH from 5.6 to 7.2 by 0.2 intervals as abscissas and milliliters of 0.01 N NH_4OH from 0 to 10.0 ml. by 1.0 intervals as ordinates.

Process. *Distillation.* To the digest obtained in the usual manner, add a slight excess of *50% nitrogen-free sodium hydroxide solution* and distil the ammonia in the Pregl apparatus into exactly 10 ml. of *4% boric acid solution*, contained in a 125-ml. Erlenmeyer flask, until the volume of the distillate measures 25 ml.

pH Measurement. Dilute the distillate to 150 ml. in a 250-ml. beaker with nitrogen-free boiled conductivity water, measure the pH, and find the corresponding milliliters of 0.01 N alkali in the graph.

XII. Other Micro Modifications. In selecting the foregoing methods, the literature has been far from exhausted. Enterprise that formerly went to devising carbon dioxide apparatus and fat extractors has recently been devoted to developing micro modifications of the Kjeldahl method, nevertheless the Kjeldahl-Willfarth method for ordinary food analysis, with the addition of selenium where haste is imperative, seems to hold its own. Distillation with boric acid with all its advantages hangs in the balance, partly because of conservatism and partly because of uncertainty as to its applicability in all cases. This hesitancy, however, does not mean that further effort is futile.

Suggestive of a possible revolutionary method is a simple procedure proposed by Jaramillo,[45] of the Upper School of Agriculture of the Republic of Colombia: A portion of the sample is placed in a copper test tube, provided with an exit tube dipping into *standard acid*, then gradually heated with 1 g. of pure *sodium hydroxide* and 2 g. of *crystalline sodium acetate.* On raising the heat, there is a copious evolution of methane, which causes a rapid bubbling through the acid, marking the end of the distillation. Finally the distillate is titrated back with *standard alkali.* The whole operation is completed in about 20 minutes.

In general micro methods are well adapted to the analysis of pure substances or biological material where the sample is homogeneous but limited in amount, whereas macro methods are usually to be preferred for ordinary food analysis where economy of labor in grinding to a degree of fineness that permits weighing a representative small charge is more essential than economy of material.

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2. CONSTITUENT GROUPS

(Water, Protein, Fat, Nifext, Fiber, Ash)

Origin of Food Analysis. The systematic analysis of foods is scarcely more than a century old. Ignoring the desultory efforts of a century previous, the establishment of the first German experiment station at Möckern by a group of landed farmers may be taken as the real start. It seems a queer violation of the order of importance that plant food (fertilizers) was given precedence over feeds, and the latter over human food. In England this early interest in plant food is explained in part by the connection of Sir John Lawes, the founder of the historic experiment station at Rothamstead, with the phosphate industry. The chief reason, however, that obtained in both England and on the Continent was that the farmers were more concerned over the fertilizers they bought to increase their crops than over the products they sold.

Fodder analysis did not lag long behind fertilizer analysis in Europe and America and in parts may have taken precedence. Human foods, however, because of their greater variety and complexity, required more preliminary research before nutrition and the food industry could be placed on the chemical basis. A glance today into a feed store and then into a modern market, displaying an almost endless variety of meats, fruits, vegetables, and groceries, suffices to show that the problems facing the food chemist and nutritionist are much more complex than those encountered by the specialists in animal feeding. The detection of adulteration, now less common than formerly, also calls for exacting analysis.

Classification of the Constituents as Six Groups. The grouping has been inherited from the early German agricultural investigators, as have been also the so-called Weende methods for their determination. These latter were successfully employed by such pioneers as Henneberg and Stohmann in Germany, a generation later by S. W. Johnson and his students, notably Atwater and Armsby, as well as others in the United States, and today by numerous investigators in animal and human nutrition the world over. They figure in law, industry, medicine, and advertising.

A complete analysis of natural vegetable products includes determination of **water, protein, fat, nitrogen-free extract** (we call it nifext), **fiber,** and **ash**; of milk all these but fiber; and of meat (except organs) all but fiber and nitrogen-free extract. All are crude figures, fiber being most often so branded.

Chemical Constituents. Each group is not made up exclusively of allied chemical substances, but rather of substances that happen to have one or more properties in common. This is even true of the loss on heating, which is not water alone, as the odor testifies. The **protein** contains, in addition to true proteins, other nitrogenous substances, such as amino acids and alkaloids; the **fat** is not merely a mixture of glycerides, but contains sterols, lecithins, and other substances of similar solubility; the **fiber** is partly cellulose and partly lignified or suberized substances; the **ash** is a mixture of common inorganic elements and may contain traces of an indefinite number of rare elements, disclosed by exacting chemical and spectroscopic methods; and finally the **nitrogen-free extract** represents, in addition to the carbohydrates, other than cellulose, and organic acids, the resultant of all the errors of the determinations of all the other constituents.

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Analytical Methods. As regards methods, not one employed for the determination of these six constituents involves precipitation, color comparison, or centrifuging, nor does one make use of distillation or titration of the substances as present in the food. The water is determined by drying at 100° in hydrogen or *in vacuo* to avoid oxidation; the protein by calculation from the total nitrogen (the final stage is, however, distillation and titration); the fat by ether extraction; the fiber by removal of all acid- and alkali-soluble constituents, and weighing the residue; the ash by incineration; and the nitrogen-free extract which is not true to name, by pure arithmetic.

The indictment that the six constituents are crude is not overstated; nevertheless the results on this century-old plan, employing methods that are (and should remain) much as first proposed, are for many purposes quite as valuable as if every constituent, known or unknown, were determined with impeccable accuracy. They are not mathematically exact, but are universally useful. They appeal to one's common sense.

a. Water or Moisture

(Loss at 100°)

Water, the simplest of all constituents of foods, is one of great concern to producer, consumer, and chemist. The weight of food has little significance unless taken into consideration with the water content.

Moisture Content of Foods. Water is everywhere and is free to all in stream and rainfall. Even when the costs of impounding and delivering through pipes are considered, it is the cheapest of all adulterants, not only for liquid products, but also for those that are moist, like bread, or even dry to the touch, such as flour that acquires moisture from the water added in tempering the grain during the milling process.

Whether naturally present, added through fraudulent intent, or present in excessive amount because of insufficient drying, water is usually a dead loss to one who buys by weight or measure. It also is an added expense for transportation if present in excessive amount. There is another side; too little water may injure the quality; vegetables lose in commercial value by wilting and bread by drying. Knowledge of the percentage of solid nutrients of foods is based on modern science, but any great excess of water or its deficiency was determined by sight and touch, even by lower animals before the appearance of man.

In cereals, dry legumes, flour, and meal the amount usually ranges from 10 to 15%. In the edible portion of succulent fruits and vegetables it may reach 95%, but in starchy vegetables, such as potatoes and fresh-shelled beans, it is commonly only 70 to 80%. Milk contains an average of 87%, lean meat and fish muscle 50 to 70%, and opened oysters 70 to 90%, fresh basis.

Determination of Water. Most of the methods for the estimation of water (moisture) in foods depend on the loss in weight on heating. The temperature employed varies from 70° for saccharine substances containing invert sugar to 110° for various foods. Commonly the temperature of boiling water is specified, the heating being carried out in a boiling water or steam oven. The temperature in such an oven even near the sea level never reaches 100°, being usually 97 to 99°; in high altitudes, such as Denver and Mexico City, it is considerably lower.

As exposure to the air of the drying oven causes the oxidation of certain oils and other constituents, a gain in weight of such constituents offsets the loss in weight due to moisture. To obviate this error the drying should be performed *in vacuo*, limiting the temperature to 70° if levulose is present, or in a current of dry hydrogen at the temperature of boiling water, the former being pre-

ferred for saccharine substances, the latter for natural foods and mill products.

The loss in weight on heating is not entirely water, as other volatile substances evident to the sense of smell are present in most foods, although the amount is usually too small to be separately determined. Most of the spices, however, contain notable quantities of volatile (essential) oil which pass off with the water. Cloves contain 15 to 25% of an essential oil, nutmegs and mace 3 to 10%, and most of the other spices smaller quantities. In these it is the common practice to determine the total loss at 110° and correct the figures thus obtained for essential oil separately determined.

Heating is not always employed to remove the water. Benedict dries at room temperature in a vacuum desiccator over sulfuric acid. Trowbridge hastens the process by gently agitating the sulfuric acid during the drying, thus mixing the surface film, which soon becomes saturated with moisture, with the lower layers.

Again, all methods do not depend on loss of weight after removal of water. The apparatus of Hoffman and of Brown and Duvel, used for the rapid determination of water in grain, are constructed so that the water driven off on heating with a petroleum oil in a flask is condensed and measured in a graduated cylinder (Fig. 96).

Hydrogen Drying Method.[1] This method is an old one. The apparatus devised by one of us has been used in the analysis of thousands of samples of cereal and oil seed products, tea, coffee, cocoa, and certain spices such as cayenne pepper, paprika, and mustard. Practically the same apparatus was adopted by Snyder for flour.[2] The results are usually higher than by the vacuum method, owing to the thorough drying of the hydrogen and the exclusion of air after drying. For drying very fatty materials, such as sausage, or moist powders, open or closed dishes are preferable.

Apparatus. Fig. 39 shows the *copper water oven* (E) and the *jars* for purifying (A) and drying (B) the hydrogen. The weighed portions of the materials are contained in glass tubes (G), preferably with glass stoppers instead of corks at both ends, which are heated in copper tubes soldered into the oven.

The *oven* is 22 cm. long, 20 cm. wide, and 18 cm. high, exclusive of the legs, which are 18 cm. long. The *copper tubes* are 17 mm. inside diameter. The *glass drying tubes* are not over 14 mm. outside diameter, fused but not flared at the end, and enter any of the copper tubes of the bath without binding. The length to the constriction is 15 cm., the total length 20 cm. A small circle ground or etched with "diamond ink" on each tube serves for a lead-pencil number or other identification mark in place of gummed labels which change in weight on heating.

A stream of *hydrogen*, purified by passing through nearly saturated sodium hydroxide solution in A and dried by sulfuric acid in B: is divided into twelve streams by means of a U-shaped metal tube with twelve offsets, one of the streams passing through each of the drying tubes. In order that the hydrogen may be evenly distributed, the mouth of each drying tube is fitted with a perforated cork through which passes an exit tube of capillary tubing with 0.5-mm. bore. The *sulfuric acid* used for drying the hydrogen falls drop by drop from the bulb C over the beads in B into the bottom of the jar, from which it automatically siphons out into D. The hydrogen passes to the bottom of the jar through a glass tube, bubbles through the acid, and rises through the beads, moist with fresh acid. The very thorough dehydration of the gas thus effected doubtless contributes to the accuracy of the results, which in flour and meal are about 1% higher (calculated as moisture) than those obtained by drying in a dish in the cell of an ordinary water oven.

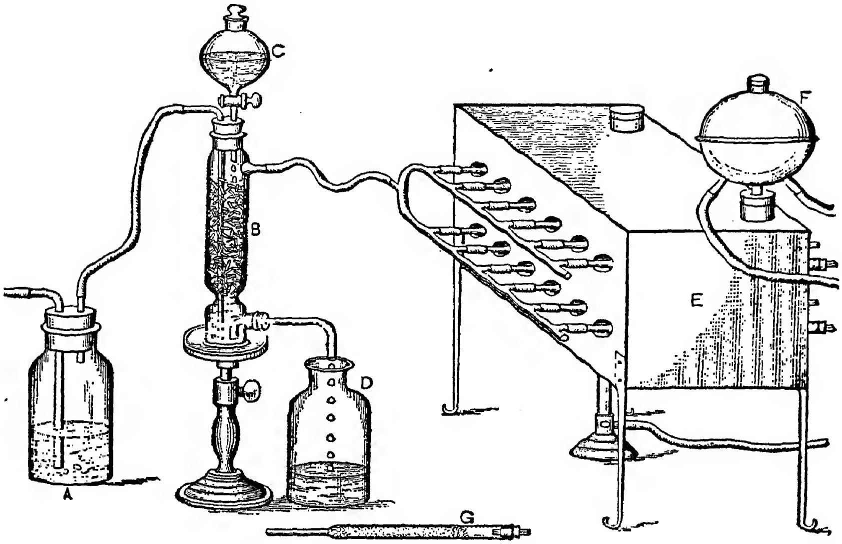

FIG. 39. Winton Multiple Hydrogen-Drying Water Apparatus.

PROCESS. The following instructions should be strictly followed to insure success.

Weighing the Charge. Place in the funnel-shaped portion of the drying tubes a small wisp of cotton weighing but a few milligrams. Although cotton contains hygroscopic moisture, the amount present in such a small quantity will not appreciably affect the results. Mix the sample thoroughly in the bottle with an aluminum spoon and weigh out 2-g. portions on a balanced watch-glass. If the watch-glasses do not weigh exactly the same, place the heavier on the left-hand pan, the lighter on the right-hand pan, and balance exactly with the rider. Introduce the weighed portions into the drying tubes through a small short-stemmed funnel of spun copper or aluminum, using a camel's-hair brush to remove the last particle from the watch-glass and funnel. Stopper and weigh the substance plus tubes, together with stoppers if glass-stoppered, but without stoppers if they are cork.

Addition of Reagents. Fill a large Kipp generator with *granulated zinc* and make up a supply of *20% sulfuric acid* ready to be introduced into the generator.

The sulfuric acid used in the drying jar can be diluted for the hydrogen generator. Place in *A* a sufficient quantity of *nearly saturated sodium hydroxide solution* to cover the lower end of the inlet tube, fill *C* with *sulfuric acid*, and see that the bath *E* contains enough water to cover the upper tier of tubes. Connect the apparatus as shown in the cut.

While the bath is heating to boiling, add the *acid* to the hydrogen generator and run for a time to expel all air, then pass the gas through *A* and *B* into the metal U-tube.

Introduction of Tube in Bath. Unstopper the glass drying tubes, insert the corks with the capillary outlet tubes, place one after another into the tubes of the drying oven, connecting each at the same time with one of the offsets of the U-tube. When all are connected, form a channel over the sub-

stance by tapping and *draw the drying tubes into the tubes of the bath until each outlet is well within the bath*, thus preventing the clogging of the capillary openings with condensed moisture, and adjust the hydrogen current so that there is a steady and moderately rapid evolution. Adjust the stopcock of *C* so that 1 drop of the acid falls over the beads in about 5 seconds.

Drying. After about 1 hour, *push the drying tubes toward the right* so that the substance is well heated and the outlet tubes project 2 to 3 cm. Test each by lighting with a match. If the capillary openings are clear, the hydrogen will ignite, usually with a distinct pop, and, when the water has been largely expelled, will burn with a miniature flame. Four hours after starting the drying is complete. Stopper each tube immediately after removing from the bath, cool 15 minutes, and weigh quickly with or without stopper as above. Keep tightly corked if the dry material is to be used for extracting with ether.

CALCULATION. Record the loss in weight and calculate the percentage of water.

NOTE FOR STUDENTS. If each of the precautions is strictly observed, the method may be carried out with facility. No difficult manipulation is involved, but attention to each detail is essential.

Spencer Vacuum Drying Method.[3] Although adopted by the A.O.A.C. for the determination of moisture in flour, the method is applicable to other dry powders and to moist substances and liquids after removal of the bulk of the water on the water bath.

APPARATUS. *Vacuum Oven*, connected with a mechanical air pump maintaining a partial vacuum of 25 mm. or less of mercury and a sulfuric acid drying bottle for admitting dry air when releasing the vacuum. The thermometer bulb should be near the sample.

Air-Tight Desiccator, containing reignited quicklime.

Metal Dish, 55 mm. high, 15 mm. deep, with slip-in cover.

PROCESS. Weigh 2 g. of the sample into the covered dish previously dried at 98 to 100°, cooled in a desiccator, and weighed at room temperature. Loosen cover and heat at 98 to 100° to constant weight (about 5 hours) in the partial vacuum of 25 mm. Admit dry air to bring to atmospheric pressure, tighten the cover, cool in the desiccator, and weigh at room temperature.

Report loss of weight as moisture.

NOTE. The cost of the apparatus is greater than that for hydrogen drying and in the writers' experience the results are somewhat lower.

Special Methods, for the determination of moisture (or solids), are given in other parts of this work.

b. PROTEIN

(Total Nitrogenous Constituents)

Protein Content of Foods. All natural foods contain protein, although there is scarcely more than a trace in honey and maple sugar. Cereals contain 8 to 15 and oil seeds as high as 50%. In fresh fruits and vegetables the amount seldom reaches 1.5%. Milk contains an average of 3.3 and lean meat and fish muscle about 20%, all on the fresh basis.

Protein Calculated from Nitrogen. The protein, or more correctly the crude protein, of foods is calculated from the total nitrogen, best determined by the Kjeldahl method as described under Part I, C1, Elemental Organic Constituents, above, by either the general factor 6.25 or a special factor such as 6.38 for milk and milk products and 5.70 for white flour and its products. These factors are at best merely approximations based on averages of composition. Each fruit, seed, root, or leaf food contains several proteins in varying proportion and of differing nitrogen content, and those of certain families or

groups contain proteins peculiar to them. Again proteins are not the only nitrogenous constituents. Amides (abundant in young shoots), ammonium salts, nitrates, lecithins (phosphatides), nucleins, nucleic acid, purines of tea, coffee, cocoa, and meat extracts, all contain nitrogen in proportions much different from those in proteins.

Notwithstanding these defects, most of which are small when the great preponderance of true proteins is considered, the protein calculated by factor is a valuable figure, not only because it represents approximately the true protein present but also because it is an index of the content of other groups. For example, high protein is usually associated in oil seeds with high oil content, medium protein in cereals and various seeds with high starch content, and low protein in sugar beets and certain fruits with relatively high sugar content.

Separation of Proteins. It is a mistake to assume that we are entirely dependent on the determination of nitrogen for all knowledge of the kind and amount of protein in foods. Fat-free muscle fibers of mammals are so nearly free of protein that physiological chemists, in despair of satisfactory chemical separations, have determined, after hydrolysis, amino acids in this material. For ages, by curdling well-skimmed milk a crude casein has been separated, and by washing white flour dough crude gluten has been obtained. The simplest of all protein separations is that of the white of egg from the yolk.

More scientific methods, employing common solvents, for the separation of proteins are described below under Part I, C4*a*, and Part II, A2.

c. Fat

(Ether Extract)

See also Part II, B1.

Fat Content of Foods. The distinction between an oil and a fat is not well marked. Cocoanut oil in the tropics is liquid, but in the temperate zone it is usually a solid fat. In the winter and in cold storage, table oils are fats. Cocoa butter, the fat of the cocoa bean and chocolate, is hard even in hot climates, hence its addition to the coating of chocolate candies. The fat of butter on a hot day is liquid when animal body fats remain solid.

High percentage of fat is usually associated with complete absence of starch, although shelled peanuts and the cocoa bean are exceptions since they contain some starch and 48 and 50% of fat respectively. Kernels of nuts, such as almonds, walnuts, and Brazil nuts, contain as high as 70% of oil; the soy bean, a starch-free legume, contains up to 20%, but starchy legumes, such as beans and peas, contain less than 3%. Maize kernels contain up to 9 and wheat up to 4% of oil located almost entirely in the germ. The amount of fat in milk on an average is 4.25, in whole hens' eggs 10.5, and in the egg yolk 33.3%. Meat contains an extremely variable amount from less than 1 to over 95%.

Separation of Fat. The oils and fats expressed from oil seeds and fruits, as well as from animal fatty tissues, correspond quite closely with those extracted by ethyl ether. In neither case is the product a pure glyceride or a mere mixture of glycerides. Practically all the sterols of the raw material and a considerable amount of the phosphorus-organic compounds, notably the lecithins (phosphatides), are expressed or extracted with the glycerides. Essential oil and resin are the chief constituents of the ether extract of certain spices. Cloves may contain over 20% of essential oil, but only about one-third that amount of true fat and resin taken together. Pepper contains 5 to 8% of a nitrogenous, ether-soluble substance, piperine, classed with the alkaloids.

Other solvents, such as chloroform, carbon tetrachloride, carbon bisulfide, and naphtha

(petroleum distillates of lower or higher boiling points), dissolve fats and oils, but the yield and composition of the extract differ somewhat with the solvent. Attempts to use them in the quantitative determination of fat have resulted in confusion, since in feeding experiments of the past hundred years ether extract has been regarded as synonymous with fat.

Dry Ether Extraction Method. APPARATUS. (*a*) *Johnson Extractor*. The form devised by Samuel W. Johnson, professor in Yale University and "father of the American experiment station," has the advantage of simplicity and small ether consumption (Fig. 40). The inner tube (*I* and *I'*) is 135 mm. long and 22 mm. outside diameter (not flanged at the top) and has a constriction at the lower end for tying on, with a strong linen thread, a piece of filter paper backed by cheese cloth. One end of the thread should be passed twice about the other and pulled taut, then the hard knot completed. After being carefully covered and trimmed once, the tube is good for a long series of determinations without renewal.

The outer tube (*O*) consists of a vertical tube 175 mm. long and 26 mm. inside diameter, with a bulge at the bottom to prevent trapping of the condensed ether, and a delivery tube attached at an angle of 45° of such a size as to permit connection, by a carefully bored cork, with the extraction flask (*F*).

A multiple apparatus for 12 determinations, devised by Professor Johnson and modified by one of the writers, is shown in Fig. 41. Steam pipes supply the heat, which is retained within the case by a glass door hung from the top on hinges. The tubes within the copper condenser tank are of block tin ⅜ in. (1 cm.) outside diameter.

(*b*) *Soxhlet Extractor*. This ingenious piece of apparatus, with a siphon that acts intermittently and automatically, is adapted for extracting quantitatively a considerable amount of material contained in a paper thimble for the further examination of the

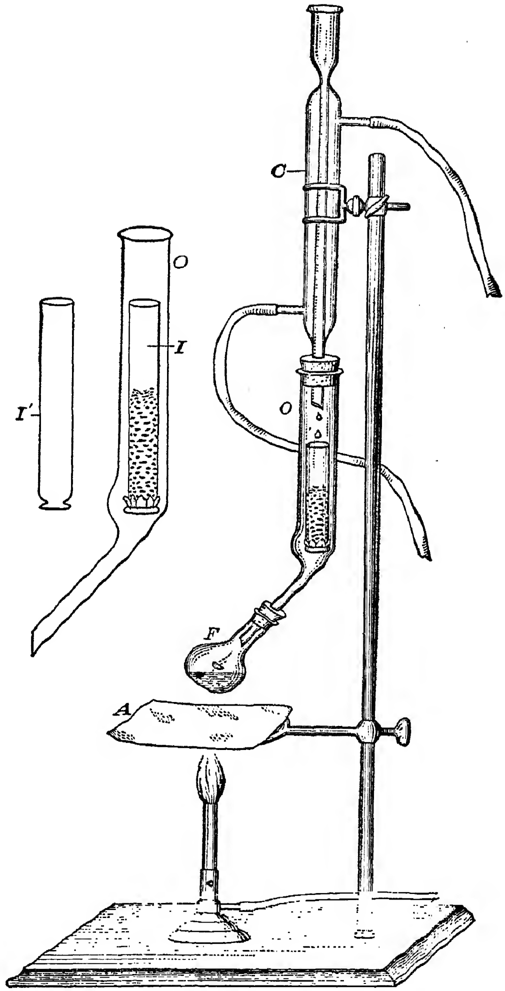

FIG. 40. Johnson Fat Extractor.

extract. Because of the large amount of ether used and the presence in many thimbles of ether-soluble matter, it is not so well

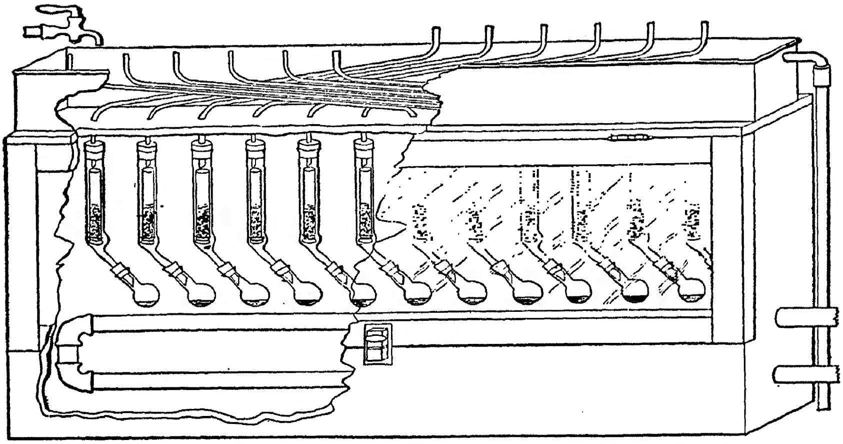

FIG. 41. Multiple Johnson Fat Extractor with Heating Closet and Condenser.

suited for ordinary analysis as the Johnson extractor is.

(c) *Mann Extractor* (Fig. 42).[4] When the substance to be extracted suffers decomposition by exposure to heat or oxidation if air is not excluded, the Mann extractor is to be preferred to the Johnson and Soxhlet extractors. The substance is contained in a bag made of ether-extracted filter paper or other material which is submerged in the solvent in V during the extraction. The vapors of the solvent volatilized in the flask F are liquefied in passing through the condenser tube causing a corresponding amount of the solvent laden with extracted matter to discharge into the extraction flask. A mercury joint prevents loss of solvent about the stopper S. For gentle heating, an incandescent bulb at H may be used. The stopcock at the upper left is connected with an air pump.

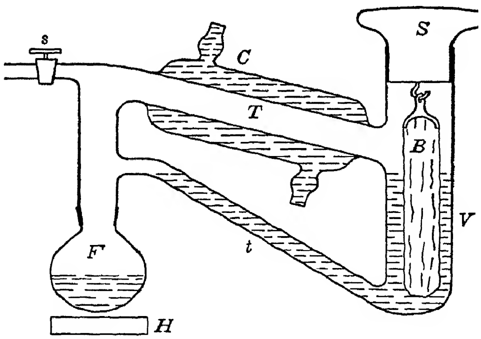

FIG. 42. Mann Cold Extraction Apparatus.

Not only fat may be extracted in the apparatus, but also sugars and other water-soluble substances at temperatures no higher than 10 to 15° above room temperature, provided the air is well exhausted.

(d) The *Knorr extractor* and (e) the *Sy Extractor* are similar to the Johnson extractor, but they are provided with mercury seals instead of corks.

REAGENT. *Ether*. Commercial ether contains a certain amount of ethanol and water; this unfits it for use as an analytical solvent. Only c.p. ether distilled over metallic sodium is suited for the purpose. It should be tested for residue.

PROCESS. *Extraction*. The material remaining after drying for the determination of

water, carefully protected against absorption of moisture from the air, is in proper condition for the extraction of fat, and the residue therefrom for the determination of fiber.

The operation of fat extraction, in which the Johnson extractor is used, is clearly shown in Fig. 40. To the lower end of the delivery tube is attached a tared 30-ml. flask with a neck of sufficient size to admit the stopper for half its length. Only 8 to 10 ml. of *ether* are required for most materials and this may be recovered at the end of the 4-hour extraction by removing the flask and substituting a test tube. The outer tube, as well as the flask, should be kept warm to bring about hot extraction with the ether that drips through the material. Any accumulation of extract in the apparatus may later be washed down by removing the heat and allowing the ether to condense on the inner surface of the outer tube and about the lower end of the inner tube.

Drying and Weighing. After the extraction for 4 hours, remove the flask, evaporate the ether at a gentle heat, and dry in a boiling water bath to constant weight.

CALCULATION. Multiply the weight of the fat by 100 and divide by the weight of the charge to obtain the percentage of fat.

Other Extraction Methods. Methods for dry and wet extraction are described in Part II, G1, J1, and J3.

d. NIFEXT *or* NITROGEN-FREE EXTRACT

(Carbohydrates, Gums, Organic Acids, Etc.)

Calculation of Nitrogen-Free Extract. Subtract the sum of the percentages of water, protein, fat, fiber, and ash from 100.

Significance of Nitrogen-Free Extract. The wisdom of the early nutritionists in classifying carbohydrates and other substances obtained by difference under the head of nitrogen-free extract is best shown by the failure of a century of critics to suggest a better course or a better name. As for the designation nitrogen-free extract, it is not so inappropriate as scientific jokers would have it. It represents approximately the starch, gums, sugars, and organic acids (all nitrogen-free) which may be extracted by water or diastase from cleaned, dried, and defatted foods. Malt extract is a comparable extract containing about 75% of solids of which all but about 5% consists of carbohydrates or true nitrogen-free extract.

The chief objection to the term is its length—three words and a total of nineteen letters—whereas the sum of the letters in the other five names of the proximate constituents (water, protein, fat, fiber, and ash) is only four letters more. In tabulation of the vast amount of data in the literature, such a cumbersome column heading is a typographical nuisance. In the *Structure and Composition of Foods*, the writers suggested a coined word *nifext*, which is really only a pronounceable abbreviation and not a step in the too prevalent change in nomenclature that accomplishes no good purpose. This has received so much favorable comment (and adverse criticism) as to embolden them to use it in subsequent tables of this work. Objectors will at least appreciate that it is not based on an erroneous assumption such as the often-used term carbohydrates applied to carbohydrates less fiber or cellulose and it is not a capital offense such as NFX would be.

e. FIBER

All the six constituents, as determined in the course of the usual proximate analysis of vegetable foods for man or beast, are crude, but all except the fiber and ash usually escape that degrading appellation. An effort to make crude ash less crude is described under Ash Analysis (Part I, C8*a*) but all attempts to purify crude fiber in chemical analysis have ended in failure because, first, no one knows what pure fiber is, as applied to food,

and, second, because results on a more solid scientific basis would be of little more value than what chemists have been blunderingly reporting during a century. It represents an indefinite sort of worthless material or roughage, bearing about the same relationship to nitrogen-free extract as sawdust does to starch. A correct numerical statement of the amount of the worthless constituents would give us a little more accurate idea of the amount of the useful nitrogen-free extract, since fiber is one of the factors in obtaining the amount of nitrogen-free extract by difference; that would be about the only advantage of the corrected over the crude figures.

A more definite statement of the crudity of the result is due. The cell wall material left after boiling with dilute acid and alkali in the process is a mixture of cellulose, lignin, and pentosans, together with sand, silica, and other mineral matter locked in the tissues, and a little nitrogenous matter. Determinations of some of these, as for example nitrogen in one of duplicates and ash in the other, are carried out by some as a special refinement of the process, but this leaves us still with no definite idea of the polysaccharide complex.

See C7 below for methods for the determination of cellulose and lignin.

The writers drop the adjective crude as applied to all six of the proximate constituents, as every chemist knows they are crude and such incrimination seems a useless recital of analytical frailties.

Fiber Content of Foods. Since fiber is cell wall material, no part of a natural vegetable substance is free from it. The amount ranges from less than 1% in some succulent fruits and vegetables to as high as 30% in cinnamon. The content in naked cereal grains seldom exceeds 3 and in wheat bran 11%. In white flour it may be as low as 0.06% including analytical errors. Nut shells and fruit stones, free from the kernels, contain as high as 60%; these, although not edible, at one time were used in powder form as adulterants for spices. Meat fibers and other fibrous organic substances are of a protein nature and not fiber in the sense here used.

Henneberg Acid-Alkali Gravimetric Method.[5] As the process is purely conventional, strict adherence to the details is highly desirable as a means of securing comparable results by different analysts in different materials and at different periods in the progress of science. It has come down to us as a heritage, essentially as here described.

REAGENTS. *Sulfuric Acid,* 1.25%.

Potassium Hydroxide Solution, 1.25%.

Ethanol.

Ether.

PROCESS. *Preliminary Treatment.* Dry in a boiling water oven for 3 or 4 hours a cylindrical weighing bottle, 75 mm. high and 40 mm. in diameter, without a constricted neck, containing a rolled-up 11-cm. filter paper pleated ready for use and bearing a lead-pencil mark corresponding to those on the bottle and its stopper (Fig. 43). A ground surface with pencil marks on both the bottle and the stopper avoids permanent marks or numbers.

Remove from the fat extractor the inner tube, containing the residue from the determination of ether extract, and allow the ether to evaporate. Empty the dry residue into an Erlenmeyer flask (Fig. 44) with a label showing the level of 200 ml. and brush out any that may adhere with a camel's-hair brush on the end of a glass rod. All this should be done on the day when the extraction is performed, as the fiber process, owing to the slow filtration after boiling with acid, may require all the time of a working day.

Acid Digestion. Heat a *1.25% sulfuric acid solution,* checked for accuracy by titration, and when the boiling point is reached immediately pour into the Erlenmeyer flask up to the 200 ml. mark. Without delay, heat the flask and contents over a gauze with a moderate-sized flame, taking care to watch the liquid constantly and lower the flame to

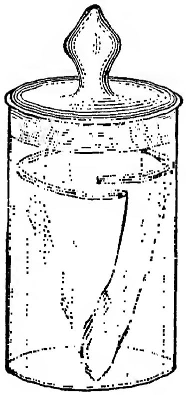

FIG. 43. Weighing Bottle with Filter Paper for Fiber Determination.

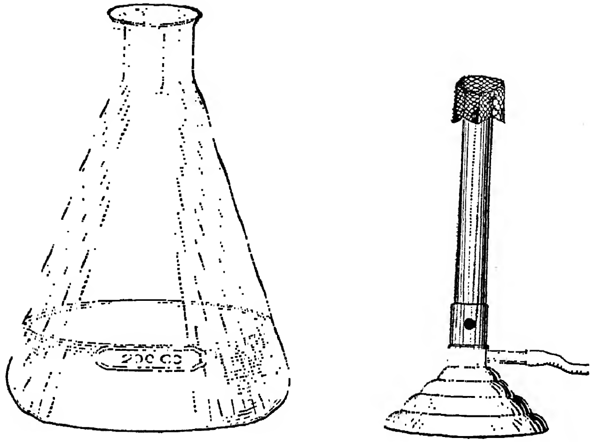

FIG. 44. Tube, Flask, and Bunsen Burner with Wire Gauze Cap for Fiber Determination.

the smallest possible size at the first indication of boiling. If the liquid is allowed to boil vigorously, it is almost sure to froth over and without warning, and thus ruin the determination.

Keep at hand a Farrington tube, which is a glass tube bent at right angles at the end (Fig. 44), so that a blast of air from the mouth can be instantly directed into the flask to prevent frothing. In order to reduce the flame to a small size without snapping down, cover the top of the Bunsen burner with a cap of wire gauze (Fig. 44). It is well to keep gauze caps on all the burners used in food work, as low flames are often desirable.

Adjust the flame so that the liquid boils very gently and continue the boiling exactly 30 minutes. If these precautions are observed, not only will there be little danger of frothing over, but also the substance will not crawl up far on the sides of the flask where it would be washed by condensed water and not subjected to the action of the acid. Any small amount that does crawl up may be brought down by very gentle rotation of the flask toward the end of the process. Concentration of the acid can thus be quite as effectually controlled as by the use of a cumbersome reflux condenser recommended by some chemists.

Acid Filtration. At the end of 30 minutes, filter without delay on a 11-cm. paper (not ashless), selecting a quality known to filter rapidly. Keep the paper well filled with liquid. If it clogs to such an extent that the filtration cannot be finished in, say, an hour, use a second paper or even a third. Rinse the flask once only, using a few milliliters of hot water, but do not attempt to remove all the substance to the paper. The water used to rinse the flask is sufficient to wash the paper.

Alkali Digestion. When the filtration is nearly finished, heat to boiling in a beaker a *1.25% sodium hydroxide solution* of accurate strength. Spread out the paper (or papers) on a 12.5-cm. funnel and rinse with the hot *alkali* back again into the flask used for the acid boiling. The alkali speedily removes the substance from the paper, leaving suffi-

cient to rinse the funnel and wash down the sides of the flask. When the level of the mark on the flask is reached, heat to boiling, and boil gently for 30 minutes exactly as in the acid boiling. As frothing during the alkali boiling is likely to take place with scarcely any warning, it is necessary to control carefully the heat and keep constant watch.

After 15 minutes' boiling, remove the weighing bottle with filter from the water oven and stopper; at the end of 30 minutes' boiling weigh. Do not cool in desiccator.

Alkali Filtration. Filter on the weighed paper, rinse all the material out of the flask, and wash thoroughly on the paper, using hot distilled water. The alkali filtration of most substances proceeds rapidly. After the washing with hot water has removed the alkali, as tested with *litmus paper*, wash twice with *ethanol* and three times with *ether*, taking care to direct the stream into the fiber, otherwise it may not penetrate the mass. Remove the funnel from the flask and keep overnight in a warm place to facilitate evaporation of the ether.

Drying and Weighing. On the next day, if there is no evidence of moisture in the fiber, carefully transfer the paper with fiber from the funnel to the weighing bottle, dry in the boiling water oven for 3 hours, stopper, cool 15 minutes, and weigh. Do not cool in desiccator.

Ash Correction. Wrap the filter paper closely about the fiber and burn to whiteness at a bright red heat in a porcelain capsule or crucible. When cool, the ash may be readily brushed off from the crucible and weighed on a balanced watch-glass. Correct the weight of this ash for any ash in the paper and deduct the weight thus corrected from the weight of the fiber.

CALCULATION. Obtain the percentage of fiber in the usual manner.

NOTE. Filtering of the acid residue on linen, as practiced in some laboratories, causes a loss of material varying with the fineness of the sample and the texture of the linen. If the amount of fiber is small, it may be finally collected and weighed in a Gooch crucible with a thin layer of asbestos.

f. ASH OR MINERAL CONSTITUENTS

See Part I, 8*a*, Scheme for Complete Ash Analysis, and methods for the determination of ash constituents.

Ash Content of Foods. Root and leaf vegetables and the pulp of fruits, although low in ash content on the fresh basis, when calculated to the dry substance may show a higher percentage than whole seeds. Parts of seeds, however, show a wider range; for example, wheat grain seldom contains as high as 3.5% of ash, but the bran may contain 8% and the germ 5%; on the other hand, patent flour may contain only 0.3 and commonly about 0.5%. Oil cakes, tea, cocoa shells, red pepper, black pepper, and undecorticated ginger and turmeric may run as high as 8 to 10% in ash, the highest figures being due to adhering soil.

Many prepared foods contain salt for seasoning. In the manufacture of glucose by the modern process, the small amount of hydrochloric acid required for hydrolysis, after neutralization, remains in the product as salt.

Oven products made with baking powder contain, in addition to salt, the residue from the baking powder or baking chemicals used for leavening.

The use of alkali in the manufacture of Dutch process cocoa increases materially the ash content and the alkalinity of the ash.

Methods for Ash Determination. Incineration merely for the purpose of determining the percentage of ash, as one of the six constituents of a complete proximate analysis, is to be distinguished from the preparation of ash in a definite amount of the material for a subsequent complete ash analysis. Instructions for the former follow.

Total Ash

Single Incineration Method. Burn 2 g. of the air-dry sample, or an amount of a moist sample containing about 2 g. of dry matter, in a platinum, quartz, or porcelain flat-bottom dish (the preference being in the order named) at a heat below redness to a white or gray ash. Larger amounts of materials containing added sugar or starch, having practically no ash, may be used so as to secure about the same amount of ash as the sugar- or starch-free substance.

A few additional suggestions may be found useful:

Substances of a woody nature, as distinguished from those which are starchy or fatty, are readily reduced to ash when heated on an asbestos paper. A piece of clean asbestos paper placed for a moment over the dish, toward the end, will aid in burning the charcoal on the surface, or the burning may be finished in a muffle furnace.

Ground seeds, particularly cereals, burn slowly toward the end of the process. White flour is particularly troublesome, although the percentage of ash is very low. Raising the heat to full redness hinders rather than helps, because the potassium phosphate fuses about the carbon particles. A muffle furnace is particularly useful in ashing such materials.

Double Incineration Method. A common procedure that is recommended for saccharine and other difficultly combustible products is as follows: Remove the heat when the carbon resists burning, cool, boil with water, filter, and return the paper and carbon to the dish for burning to whiteness which may be at a red heat Add to the residue the filtrate, evaporate to dryness, and ignite cautiously to prevent decrepitation at very dull redness.

A shorter course that often is adequate is to evaporate after adding the water without filtering, depending on the distribution of the disintegrated carbon over the bottom of the dish to make full use of the heat.

If other means fail, cooling and adding a drop of *nitric acid*, or *ammonium nitrate or acetate solution*, repeating if necessary, insures rapid combustion without introducing significant error, especially if the ash is neutral. Special methods are given for flour (Part II, A2) and other difficultly incinerated substances.

Water-Insoluble Ash

(Insoluble Ash)

Direct Method. Add to the ash 50 ml. of water, heat to boiling, collect the insoluble matter in a tared Gooch crucible, wash with hot water, dry, heat below redness, cool in a desiccator, and weigh.

Water-Soluble Ash

(Soluble Ash)

Indirect and Direct Methods. Obtain by difference, subtracting the weight of the insoluble ash from that of the total ash, or evaporate the filtrate from the insoluble ash to dryness in the dish used for the burning and cautiously ignite below redness.

Alkalinity of Total Ash

Indirect Method for Ash Rich in Carbonates. Remove the ash obtained as above directed to a beaker, using 100 ml. of water to rinse the dish, add an excess of *standard 0.1 N hydrochloric or sulfuric acid*, then cover with a watch-glass and boil down to at least half the volume, thus insuring complete removal of carbon dioxide. Cool and titrate the excess of acid with *standard 0.1 N sodium or potassium hydroxide solution*, using *phenolphthalein* as indicator. Subtract the number of milliliters of alkali used in the titration from the number of milliliters of acid first added, thus obtaining the number of milliliters of acid corresponding to the grams of

the sample ashed. Express the result as the number of milliliters of 0.1 *N* acid per gram of the sample, or milliliters of normal acid per 100 g. of the sample, or in such other form as official ruling or custom may dictate.

Direct Method for Ash Containing Little or No Carbon Dioxide. Add to the ash, obtained as directed for Total Ash above without removal from the dish, about 50 ml. of water and titrate directly with *standard 0.1 N acid,* using *methyl orange* as indicator. Report as above.

Alkalinity of Soluble and Insoluble Ash

Titration Method. Determine the alkalinity of the soluble ash in the filtrate from the insoluble ash. (For this purpose filtering and washing on paper are preferred.) Obtain the alkalinity of the insoluble ash by subtracting the alkalinity of the soluble ash from that of the total ash.

Acid-Insoluble Ash

(Sand)

Acid Digestion Method. Burn an amount of the sample equivalent to 2 to 5 g. of dry matter as for the determination of total ash, but without attempting to remove the last traces of carbon. Boil the ash with 25 ml. of *10% hydrochloric acid* (sp.gr. 1.050), filter on a Gooch crucible, wash with hot water, ignite at full redness, and weigh.

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[2] *Ind. Eng. Chem.* 1926, **18**, 272.
[3] *J. Assoc. Official Agr. Chem.* 1925, **8**, 665; 1926, **9**, 39; Official A.O.A.C. Method for Flour.
[4] *J. Biol. Chem.* 1920, **44**, 207.
[5] *Landw. Vers.-Sta.* 1864, **6**, 497.

3. WATER

See also Part II, A1, A2, and E3.

Water is the complement of solids. In solid or semi-solid foods the results are expressed as water (moisture), in liquids and viscous substances, as solids or dry matter. The frequent use of the term total solids is unfortunate. It doubtless was introduced as a reminder that in most foods the solids are made up of several constituents although the plural form conveys the same idea.

Temperature of Drying. As noted in the previous section, water is commonly determined by drying, usually at or near the temperature of boiling water. The results are often expressed as loss at 100°, 97°, or such other temperature as the thermometer in the drying cell indicates. In air ovens with electric regulators constant temperature of 100° or other suitable temperature may be attained.

Errors of Drying Method. The loss at 100°, or rather the temperature of boiling water, as stated in C2*a* above, is not always pure water. The ethanol of brandied peaches and the volatile (essential) oil of spices are driven off at that temperature; on the other hand, the last traces of water are removed with difficulty or moisture may be taken up between the drying and the weighing. Oxidation causes a gain in weight. For many common foods, drying in the water oven, with suitable precautions, gives results sufficient for practical purposes. The methods described under Spices (Part II, J1) obviate the error due to the presence of volatile oil.

Calculation of Water Content (Solids) from Physical Measurements. When the solids of liquid or viscous food consists essentially of a single substance, such as sucrose or lactose, the percentage of solids may be derived from the specific gravity by a formula or table based on a formula or actual determinations. Even when other constituents are also present, although in smaller amount, as in molasses or sirups, such a calculation may yield results sufficiently accurate for practical purposes.

Calculation of Solids from Mixed Data. In milk the solids or solids-not-fat may be calculated with a reasonable degree of accuracy from the specific gravity and the percentage of fat. Several formulas have been proposed and tables derived therefrom have been published. See Part II, G1.

4. PROTEIN

The term protein, as used throughout this work, refers to the group; the term proteins refers to the pure substances.

The percentage of protein, as calculated by factor 6.25 from the percentage of nitrogen, represents one or more undetermined proteins, also error due to the variation in the true factor, as well as in the determination of nitrogen; the latter error if 0.1% becomes 0.625% when multiplied by the factor. For some purposes the result is expressed in terms of nitrogen, for others in terms of protein. Single or grouped proteins may be regarded as representing not proteins alone, together with impurities and error, but also as a complex of constituent amino acids just as fat is known to consist of a mixture of different glycerides, sterols, and phosphatides, the difference being, however, that in a protein the amino acids are probably united in one molecule, whereas in a fat the constituents exist largely as individuals.

a. Pure or True Proteins

The following tests for proteins differ in their specificity since they depend on the presence of certain amino acids or distinctive groups.

Tests for Proteins. **Piotrowski Biuret Test.** Mix 1 ml. of the solution with an equal volume of *10% sodium hydroxide solution*, then add a few drops of *1% cupric sulfate solution*, stirring thoroughly after each addition. The color formed on standing a few minutes in the presence of proteins and many protein derivatives varies from blue to pink according to the length of the chain of —CONH— groups.

Millon Mercuric Nitrate-Nitrite Test. Reagent. Dissolve metallic mercury in an equal weight of fuming nitric acid and dilute with an equal volume of water. The solution contains mercuric nitrate together with mercuric nitrite that reacts with the proteins. A solution of mercury in ordinary nitric acid is not active.

Process. Heat the solution of the unknown with a small quantity of the *reagent*. The brick red coloration obtained in a positive test is due to the presence of tyrosine in the protein and is really a test for the hydroxyphenol group, which may occur in substances other than proteins.

Xanthoproteic Test. Treat the sample with *nitric acid*. Proteins give a distinct yellow color which changes to orange with *ammonium hydroxide*.

Hopkins-Cole Glyoxylic Acid Test. The violet color formed in the Adamkiewicz acetic-sulfuric acid test has been shown to be due to the presence in the acetic acid of glyoxylic acid which breaks down into an aldehyde. In the Hopkins-Cole test a solution of glyoxylic acid is employed. The test depends on the presence of tryptophan in the protein.

Reagent. Treat 1 g. of powdered magnesium with 25 ml. of saturated oxalic acid solution with cooling. Filter to remove the magnesium oxalate, add acetic acid to acid reaction, and dilute to 100 ml.; preserve with a few drops of chloroform.

Process. Mix equal volumes of the unknown and the *reagent* and underlay with *sulfuric acid*. A red-violet color forms on standing.

Stutzer Cupric Hydroxide Method.[1] This method was designed to separate the true proteins from amino and other non-protein

nitrogenous substances. It has wide application in agricultural laboratories and in the opinion of Fraps and Bizzel,[2] is more satisfactory for vegetable materials than the phosphotungstic acid method.

REAGENT. *Stutzer Reagent.* Dissolve 20 g. of recrystallized $CuSO_4 \cdot 5H_2O$ in 1 liter of water, add 0.5 ml. of glycerol, and precipitate the cupric hydroxide with dilute NaOH solution added to slight alkaline reaction. Filter, rub in a mortar with water containing 0.5% by volume of glycerol, filter by decantation, and wash on the paper until the washings are no longer alkaline. Rub up the precipitate with 10% glycerol solution and calculate the weight of copper hydroxide per milliliter of the suspension.

PROCESS. Prepare the sample by drying, if necessary, and grinding. Weigh out a quantity equivalent to about 0.7 g. of dry matter, add 100 ml. of water, and heat slowly to boiling on a piece of asbestos paper with constant stirring. If the substance is a seed or seed product such as oil cake, decompose the alkaline phosphates by adding a few milliliters of *concentrated potash alum.* Add from a pipet a volume of *Stutzer reagent* equivalent to about 0.5 g. of copper hydroxide, stir well, allow to cool, filter, and wash with cold water. Determine the nitrogen in the precipitate without removing from the paper, adding *potassium sulfide solution* sufficient to precipitate the copper before distilling the ammonium hydroxide.

CALCULATION. Obtain the protein by the factor 6.25 or a special factor.

Barnstein Modification.[3] This process is simpler than the original Stutzer method and gives satisfactory results. It differs merely in that the material is heated with only 50 ml. of water and, instead of suspended copper hydroxide, 25 ml. of *cupric sulfate solution* (6 g. of recrystallized salt per 100 ml.) and 25 ml. of *1.25% sodium hydroxide solution* are added. After filtering, the washing is continued until potassium ferrocyanide or calcium chloride solution no longer forms a precipitate.

Schjerning Uranium Acetate Method.[4] After numerous experiments on the precipitation of proteins in beer wort and in cereal, oil cake, and animal products, Schjerning found that either *uranium acetate* or *phosphotungstic acid* precipitates all the proteins, and that the same is true of *cupric hydroxide,* at least as regards the proteins of cereal and oil cake products, whereas *stannous chloride, lead acetate, mercuric chloride, ferric acetate, magnesium sulfate, bromine, tannic acid,* and some other reagents act as precipitants for rather indefinite groups. *Phosphotungstic acid* precipitates, in addition to proteins, various amines, amino acids, and alkaloids, often in large amount; *cupric hydroxide* also precipitates some non-protein substances, but not to so great an extent as to appreciably affect the accuracy of the Stutzer method as applied to foods. As *uranium acetate* precipitates, in addition to the proteins of foods, only ammonia, Schjerning chose this reagent for his method, proceeding as follows.

PROCESS. *Uranium Precipitation.* Digest 0.5 to 1.0 g. of the material in a beaker with 100 ml. of water at room temperature for 20 hours with frequent stirring, heat on a water bath to 50°, add an excess (20 to 40 ml.) of *saturated uranium acetate solution,* and hold at 50° for 30 minutes in a dark place. As sodium-free uranium acetate is apt to be basic, it is better to use the grade prepared for the determination of phosphoric acid, such as Merck's, which contains the sodium as well as the uranium salt. Filter on a 11-cm. paper (hydrochloric acid extracted) and wash two or three times with *1 to 2% uranium acetate solution.*

Ammonia Distillation. Place the precipitate and filter in a Kjeldahl flask with 50 ml. of *milk of magnesia* (5.5 g. of *magnesium oxide* per liter of water) and evaporate nearly to dryness on an asbestos board to

remove ammonia, then add *sulfuric acid* and proceed according to the Willfarth modification. Correct for the solubility of the uranium precipitate by adding 0.1 ml. of 0.1 *N* acid for each 100 ml. of filtrate and washings.

Digestible Proteins

Stutzer Pepsin Method.[5] Stutzer, who first devised a practicable method of digestion *in vitro*, used artificial *gastric juice*, prepared from hog's stomach, containing 0.2% of *hydrochloric acid*. The digestion was continued 48 hours at 40°, adding from time to time *10% hydrochloric acid* until the content in the liquid reached 1%. To further imitate the digestive process, the material insoluble in pepsin was sometimes treated with *alkaline pancreatin solution*.

I. Wedemeyer Modification.[6] This simplification of the original process employs a solution of commercial *pepsin in dilute hydrochloric acid* and is commonly preferred to the original method. *Pepsin solution* containing *0.5% hydrochloric acid* is added at the start, increasing to 1% at the end of the twenty-fourth hour. As the addition of acid in several portions entails little more labor, it seems best in this detail to adhere more closely to the original process.

II. Sjollema Modification.[7] Digest 2 g. of the material in a beaker with 430 ml. of water, 1 g. of *pepsin* (standard quality), and 16 ml. of *10% hydrochloric acid* for 48 hours at 38 to 40° with repeated stirring. At the end of the sixteenth, twenty-fourth, and fortieth hours, add 11 ml. of *10% hydrochloric acid*. After cooling, filter, wash with warm water, and determine the nitrogen in the residue by the Kjeldahl-Willfarth method, thus obtaining the indigestible nitrogen. The digestible nitrogen is obtained by difference.

Coagulable Proteins, Proteoses, and Other Animal Proteins

See Part II, H1 and H3.

Protein (Nitrogen) Distribution

Hausmann Phosphotungstic Acid Comprehensive Method Modified by Osborne and Harris.[8] The original method was for three groups (ammonia, basic nitrogen, and nonbasic nitrogen) to which the American contributors added a fourth (humin nitrogen).

Process. *Hydrolysis*. Subject a weighed charge of the protein, equivalent to about 1 g. of dry matter, to hydrolysis by refluxing for 5 hours, with 20 ml. of *hydrochloric acid*.

A. Ammonia (Acid Amide) Nitrogen. Remove from the hydrolyzate the excess of acid by distillation with *magnesium oxide*, or preferably *calcium hydroxide suspension*, into *standard 0.1 N sulfuric acid*, and titrate.

Calculate the percentage of nitrogen as ammonia. This ammonia is considered to be formed from $CONH_2$ linkages but Gortner and Holm [9] have shown that during the usual 24-hour hydrolysis about 20% is due to deamination of monoamino acids and that to obtain the true amount the hydrolysis should be limited to a much shorter period.

B. Humin Melanin Nitrogen. The formation during acid hydrolysis of humin as a black amorphous substance, as shown by Gortner and collaborators,[10] is due to the concentration of tryptophan with an aldehyde, although the amount formed is not a quantitative measure of the tryptophan content. Filter, wash, and determine nitrogen in the humin and paper, correcting for nitrogen in the latter.

C. Basic Nitrogen. Acidify with *hydrochloric acid* the filtrate from the humin nitrogen, evaporate to small volume, precipitate with *phosphotungstic acid solution*, and allow to stand 24 hours. Filter, wash

with a *dilute phosphotungstic acid solution* in *dilute hydrochloric acid* until the filtrate is no longer yellow. Transfer the precipitate and filter to a volumetric flask, dissolve in *sodium hydroxide solution*, make up to the mark, filter, and determine nitrogen in an aliquot of the filtrate. Multiply by the dilution factor, thus obtaining the nitrogen of the bases (arginine, histidine, lysine, and part of cystine).

D. NON-BASIC NITROGEN. Make the filtrate from the phosphotungstic precipitate up to the mark in a volumetric flask and determine nitrogen in an aliquot. Multiply by the dilution factor, thus obtaining the non-basic nitrogen (monoamino monocarboxylic and monoamino dicarboxylic acids).

Van Slyke Phosphotungstic Acid Comprehensive Method.[11] The method, or rather assembly of methods, as here described includes certain improvements generously communicated by Dr. Van Slyke. In its earlier stages it is similar to the Hausmann method, but is more carefully elaborated. In its later stages it employs (1) a method for the determination of arginine based on the discovery of Osborne, Leavenworth, and Brautlecht that arginine slowly loses half its nitrogen on refluxing with alkali, (2) application of the Benedict and Denis sulfur method to the solution of hexone bases to estimate the cystine, (3) the Van Slyke gasometric method for the determination of amino nitrogen, (4) calculation of the histidine nitrogen as 1.5 times the non-amino nitrogen of the bases found in excess of the non-amino nitrogen [the three nitrogen atoms of arginine in the guanidine group, $NH_2C : N(NH)$, yield no free amino nitrogen by the Van Slyke nitrous acid method]; and (5) estimation of lysine by the excess of NH_2 groups over carboxyl groups in the hexone base fraction, the carboxyl groups being determined by the ninhydrin-CO_2 method of Van Slyke, Dillon, MacFadyen, and Hamilton below.

The method has proved of great value in the study of pure proteins and has been widely applied in the analysis of foods, although sometimes without due regard for the interference of non-protein constituents.

APPARATUS. *Assembly for Refluxing and Evaporation under Diminished Pressure.*

Folin Bulb and *Condenser.*

REAGENTS. As for the Hausmann Method above, also the following.

Calcium Hydroxide Suspension, 10%.

Phosphotungstic Acid Wash Liquid. Use 2½% 12-phosphotungstic acid in 0.25 *N* HCl.

Denis Reagent. Dissolve 25 g. of (
$3H_2O$, 25 g. of NaCl, and 10 g. of
in water and make up to 100 ml.

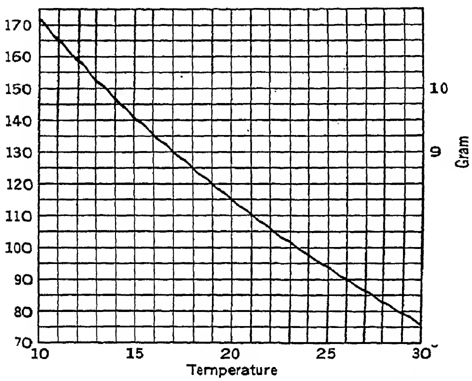
12-Phosphotungstic Acid. Commercial phosphotungstic acid is a mixture of compounds, of which the 12-acid, $H_3(PO_4 \cdot 12WO_3)$, may vary from 30 to 80%. Only the 12-acid should be used. It may be prepared as described by Wu.[12] It is usually simpler to prepare it from commercial phosphotungstic acid by the method of Winterstein.[13] The commercial product is dissolved in an equal weight of water in a separatory funnel and an equal volume of ether is added. The ether forms with the acid a heavy oil which settles below the water solution. The oily ether solution is then washed three times with each time a volume of water equal to the volume of the ether solution. The washed ether solution is then dried on a steam bath.[14]

PROCESS. A. HYDROLYSIS. Reflux about 3 g. of protein with 50 ml. of 6 *N hydrochloric acid* for 24 hours.

B. TOTAL NITROGEN DETERMINATION. Transfer the hydrolyzate to a 200-ml. double-necked distilling flask and concentrate under diminished pressure until as much as possible of the free acid has been driven off, but not so far that the residue hardens and becomes difficult to redissolve in water. Dissolve the residue in water and wash into a 100-ml.

measuring flask. Fill to the mark and take 3 aliquot portions of 5 ml. each for Kjeldahl nitrogen determinations.

C. Ammonia Determination (Amide Nitrogen). Wash the remaining 85 ml. of hydrolyzate into a 1-liter Claisen double-necked distilling flask with 100 ml. of wash water. Add 100 ml. of *ethanol* to prevent foaming, then *10% calcium hydroxide suspension* until an excess is present, as shown



Courtesy of the Authors and J. Biol. Chem. 1942, **146,** *156*

Fig. 45. Van Slyke, Hiller, and Dillon Graph for Nitrogen Distribution Method.

by turbidity and alkaline reaction. Connect with a side tube flask, cooled by a stream of water from the tap, containing about 60 ml. (vegetable proteins) or 30 ml. (animal proteins) of *0.1 N sulfuric acid,* and this in turn with a small flask of the same type containing 20 ml. of the *0.1 N acid,* connected with the exhaust. Heat the Claisen flask in a water bath at 45 to 50°, letting in a little air through the stopcock if necessary to prevent boiling over at the start, and distil for 30 minutes under diminished pressure. At the end of the distillation, lift the flask from the bath and release the pressure by opening the cock.

Wash the diluted standard acid from both flasks into a beaker and titrate with *0.1 N alkali.* Calculate the total weight of nitrogen as ammonia absorbed.

D. Humin (Melanin) Nitrogen. Collect the humin precipitated during the hydrolysis and absorbed during the ammonia determination on a pleated paper, wash free from chlorine, digest the precipitate and paper with 35 ml. of *Kjeldahl sulfuric acid,* and determine the nitrogen.

E. Separation of the Bases. (*a*) *Precipitation with 12-Phosphotungstic Acid.* Neutralize the filtrate from the humin with *hydrochloric acid,* return to the double-necked distilling flask, and concentrate to about 100 ml. Then wash into a 500-ml. Erlenmeyer flask and dilute up to the volume indicated by Fig. 45,[15] according to the room temperature at which the flasks will stand during the precipitation of the bases. For example, if the amount of hydrolyzate present after removal of samples for total nitrogen represents 2.5 g. of protein, and the precipitation is to be done at 20°, the volume used for the precipitation will be $2.5 \times 115 = 287$ ml. For each 100-ml. volume of solution add 2 ml. of *hydrochloric acid,* bringing the acid concentration to approximately 0.25 *N.* Add 5 g. of *phosphotungstic acid* for each 100 ml., and then 5 g. additional; e.g., in the above case, add 20 g. of *phosphotungstic acid.* Heat the solution until the precipitate of the bases nearly or quite dissolves; then cool and let stand 24 hours at room temperature.

(*b*) *Filtration and Washing of the Precipitate of Bases.* Collect the precipitate on a hardened filter paper in a 3-inch Büchner funnel. Wash the precipitate five times, using each time about 10 ml. of ice-cold *phosphotungstic wash liquid.* Break up the lumps with the first washing and suck dry at each washing. If cracks form, push them together during the next washing.

(*c*) *Recrystallization of the Precipitate.*[16] To remove the last traces of mono-amino acids it is necessary to recrystallize the pre-

cipitate. Transfer the precipitate back to the flask in which it was formed and suspend in the same volume of water used in the first crystallization. For each 100 ml. of water add 2 ml. of *hydrochloric acid* and 5 g. of *phosphotungstic acid*, heat to boiling, and let stand again at room temperature for 48 hours. Wash as before.

(*d*) *Removal of Phosphotungstic Acid from the Precipitate by Amyl Alcohol-Ether Mixture.*[17] The precipitation of the phosphotungstic acid with barium chloride, as used in the original method, has given place to the following treatment suggested by Jacobs.[18]

Remove the precipitate from the filter with a spatula and wash into a half-liter separatory funnel with 200 to 300 ml. of water. Add 5 to 10 ml. of *hydrochloric acid* and shake for 1 or 2 minutes with *1 + 1 amyl alcohol-ether mixture* in sufficient amount (usually 100 ml.) to float on the aqueous layer after the precipitate has dissolved. If the boundary between the solutions is not distinct, owing to humin precipitated with the phosphotungstates, pass the entire liquid through a Büchner funnel with suction; then the separation should be complete. Make three more extractions, using each time a volume of the *mixture* about one-quarter of that of the aqueous layer. Finally combine the extracts, shake once with water to remove traces of bases, then shake this water solution once or twice with the *amyl alcohol-ether mixture* and add to the main aqueous solution. Add a few drops of the aqueous solution to a *saturated barium hydroxide solution* in a small test tube; if no precipitate forms, the phosphotungstic acid has been completely removed.

Place the combined aqueous phosphotungstates-free solution of the bases in a 500-ml. double-necked distilling flask and concentrate to dryness to drive off free acid. Wash the residue with water into a 50-ml. volumetric flask and make up to volume the *solution of the bases.*

F. Filtrate from the Bases. Combine the filtrates and washings from the two crystallizations of the hexone base phosphotungstates and add saturated *sodium acetate solution* until the solution no longer turns Congo paper blue. Then concentrate the solution by distillation in a double-necked distilling flask under diminished pressure and bring to 250 ml. volume.

G. Arginine Nitrogen. The procedure depends on the observations of Osborne, Leavenworth, and Brautlecht[19] that boiling arginine with dilute alkali liberates half of its nitrogen as ammonia.

Place the 25-ml. aliquot of the solution of the basic amino acids in a 200-ml. pear-shaped flask fitted to the lower end of a condenser tube. In a Folin bulb tube connected with the upper end of the condenser tube, place 15 ml. of *0.1 N sulfuric acid* together with *alizarin sulfonate indicator*. Add to the solution in the pear-shaped flask 12.5 g. of *solid potassium hydroxide* and a few bits of *porous porcelain*, then connect with the condenser and boil gently for exactly 6 hours. Disconnect the Folin tube, pour the contents into a Kjeldahl receiver, rinsing with 100 ml. of water. Distil as in the Kjeldahl process the solution remaining in the pear-shaped flask, which contains traces of ammonia, into the solution from the Folin tube.

Titrate the distillate with *standard 0.1 N alkali;* 1 ml. = 0.0028 g. of the arginine nitrogen decomposed by the boiling with the alkali or 0.0056 g. of the total arginine nitrogen in the solution of the bases.

If cystine is present in the solution of the bases, 18% of its nitrogen is evolved as ammonia during the arginine determination, but the amount, except in keratin, is usually inconsiderable (see Cystine below).

H. Total Nitrogen of Bases. Transfer all the solution remaining in the pear-shaped flask after the arginine determination to a Kjeldahl flask, add 35 ml. of *sulfuric acid*

slowly with cooling, and 0.25 g. of *cupric sulfate*. Digest and determine the nitrogen by the Kjeldahl method; 1 ml. of the standard 0.1 N acid = 0.0028 g. of total nitrogen in the solution of the bases.

If desired a new but necessarily smaller aliquot of the basic amino acid solution may be used for the determination of the total nitrogen of the bases.

I. CYSTINE BY BENEDICT-DENIS METHOD.[20] Evaporate to dryness on a water bath in a porcelain dish, 7 to 10 cm. in diameter, 6 ml. of the solution of the basic amino acids together with 5 ml. of *Denis reagent*. Heat to redness and hold at that temperature for some minutes. Dissolve the residue in 10 ml. of *10% hydrochloric acid* and dilute to 150 ml. Heat to boiling and add 10 ml. of *5% barium chloride solution*, which must be in excess. Collect the barium sulfate on a filter, wash, ignite, and weigh in the usual manner; 1 mg. of barium sulfate = 0.06 mg. of cystine nitrogen in the solution analyzed or 0.3 mg. in the solution of total basic amino acids. Correct for sulfur in the reagent; it must not exceed 1.5 mg. Less than half of the cystine is in the portion analyzed, since about half is altered in hydrolysis to the *d-l* form not precipitated by the 12-phosphotungstic acid, and about 15 mg. of *l*-cystine nitrogen per liter remains in the filtrate because of solubility of the phosphotungstate.

J. AMINO NITROGEN OF BASES BY VAN SLYKE NITROUS ACID GASOMETRIC METHOD. Determine in a 10-ml. aliquot of the basic amino acid solution by the Van Slyke Gasometric Method below. Continue the action for 30 minutes at 20° or longer. Conduct a blank determination and correct the result accordingly. Correct also for the excess of 10% of the gas yielded by cystine. This correction is usually negligible, except in the case of keratins.

K. CARBOXYL NITROGEN OF BASES BY THE NINHYDRIN-CO_2 METHOD OF VAN SLYKE, DILLON, MACFADYEN, AND HAMILTON.[21] If the apparatus for the carboxyl nitrogen determination, below, is available, it is desirable to perform this determination, as it permits a more accurate calculation of the lysine than the original method. Place 1 ml. of the solution of the bases in the reaction vessel of the ninhydrin-CO_2 apparatus and add 1 ml. of 2 M *phosphoric acid* (1 volume of sirupy H_3PO_4 plus 6.5 volumes of water). Carry out the reaction as described on pages 80 to 84, using 100 mg. of *ninhydrin* and a heating period of exactly 8 minutes. If the protein sample is unusually high in bases, the CO_2 pressure may, in rare cases, be too high to read on the manometer. In such a case dilute a portion of the base solution with an equal volume of water and repeat the determination with 1 ml. of the diluted solution.

L. HISTIDINE NITROGEN. Calculate the grams of histidine nitrogen (H) by the formula:

$$H = 3/2(N - 3/4A) = 1.5N - 1.125A$$

in which N is the grams of non-amino nitrogen of the bases and A is the grams of arginine nitrogen.

An alternative procedure to this indirect estimation of histidine is recommended by Van Slyke, Hiller, and Dillon as more exact. This procedure is the direct colorimetric determination of histidine by the diazo method of Jorpes.[22] For this estimation enough of the hexone base solution is used to contain from 0.002 to 0.015 mg. of histidine nitrogen. Ordinarily a suitable volume of the hexone base solution can be readily calculated.

M. LYSINE NITROGEN. (*a*) If the carboxyl nitrogen has been determined calculate the lysine nitrogen (L) as follows:

$$L = 2(NH_2 \cdot N - COOH \cdot N)$$

in which $NH_2 \cdot N$ is the amino nitrogen and $COOH \cdot N$ is the carboxyl nitrogen of the bases.

(*b*) If the facilities for the carboxyl nitrogen determination are not available, calculate the lysine by the original formula of Van Slyke:

$$L = T - (A + C + H)$$

in which A is the arginine nitrogen, C the cystine nitrogen, and H the histidine nitrogen.

N. TOTAL NITROGEN IN THE FILTRATE FROM THE BASES (the Monoamino Acid Fraction). Take duplicate 25-ml. portions from the 250 ml. of the filtrate for Kjeldahl nitrogen. For each portion use 15 g. of *potassium sulfate,* 35 ml. of *sulfuric acid,* and 0.25 g. of *cupric sulfate.* Add the *sulfuric acid* carefully under a hood, because evolution of hydrochloric acid is vigorous. Continue the digestion for 3 hours after the solution has become clear.

O. AMINO NITROGEN OF THE FILTRATE FROM THE BASES. Use for the determination of the amino nitrogen 10-ml. portions of the filtrate and run in the usual manner for 5 minutes at 20°, 4 at 25°, or 3 at 30°.

P. CORRECTIONS FOR SOLUBILITIES OF THE PHOSPHOTUNGSTATES OF THE BASES.[23] When the phosphotungstates are precipitated and washed under the above conditions, the following amounts escape into the filtrate: arginine nitrogen equal to 0.6% of the total protein nitrogen; lysine nitrogen equal to 0.3% of the total; histidine nitrogen, variable but ordinarily about 0.6% of the total. For example, if the total nitrogen in the hydrolyzate (after removal of 15/100 for Kjeldahl) is 400 mg., the arginine nitrogen escaping into the filtrate will be 2.4 mg.

b. AMINO ACIDS

In this section are described the methods for the determination of the amino acids which have been isolated from proteins. Methods for glutathione, a tripeptide, are also included.

TOTAL AMINO ACIDS

Sørensen Formol Volumetric Method.[24] This important method is based on the formation of acid compounds by the action of formaldehyde on neutral amino acids. The amino groups are fixed as methylene derivatives or similar products thus:

$$- CH_2{\cdot}O \rightarrow$$
$$CH_2{:}N{\cdot}RCH{\cdot}COOH \quad {\cdot}\, H_2O$$

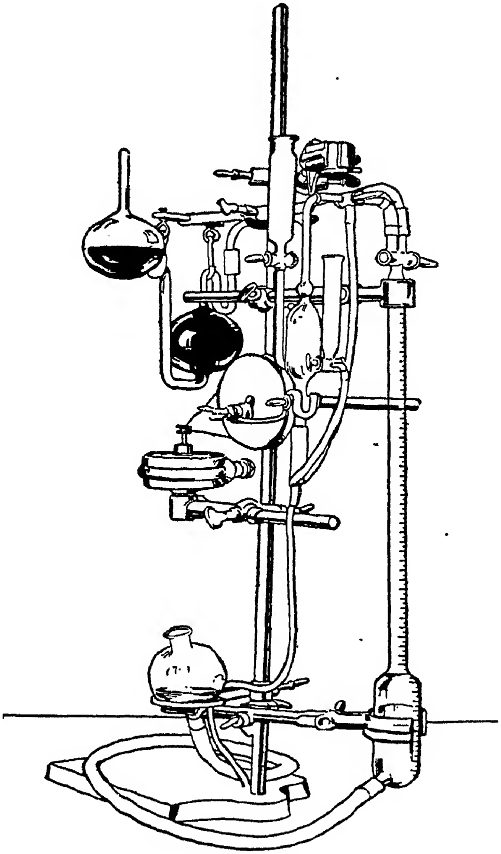
After the fixation, the carboxyl groups may be titrated with 0.2 N alkali; hence the method measures the carboxyl groups, whereas the Van Slyke method measures the amino groups.

REAGENT. *Neutral Formaldehyde Solution.* Neutralize 37% formaldehyde until faintly red to phenolphthalein.

CONTROL TITRATION. Mix 50 ml. of boiled water and 20 ml. of *neutral formaldehyde solution,* add 5 ml. of *standard 0.2 N sodium hydroxide (or barium hydroxide) solution* free from carbonates, and titrate with *0.2 N hydrochloric acid,* using 1 ml. of *phenolphthalein solution* as indicator, until a faint red color appears, then add 3 drops of *saturated barium hydroxide solution* to form a decided red coloration.

PROCESS. *Solution.* Weigh a quantity of the material, or pipet an aliquot of a cold water solution, equivalent to about 2 g. of dry matter, into a 100-ml. volumetric flask and dilute with 50 ml. of water. Add 1 ml. of *phenolphthalein solution* and 10 ml. of *10% barium chloride solution.* Titrate with *saturated barium hydroxide solution* until a red color appears, then add an excess of about 5 ml. Make up to the mark, shake, allow to stand 15 minutes, and filter through a dry paper. Remove an aliquot of 80 ml., distil the ammonia in a vacuum apparatus, and add to the residue a little *hydrochloric acid* to bring insoluble matter in solution. Pass carbon dioxide-free air through the solution to remove carbon dioxide and

neutralize carefully, first with *carbon dioxide-free sodium hydroxide solution* until delicate litmus paper is only faintly blue and finally with *0.2 N hydrochloric acid.*



Courtesy of the Author, the J. Biol. Chem., and the Methods of Analysis of the A.O.A.C.

FIG. 46. Van Slyke Amino Nitrogen Assembly.

Titration. To the ammonia-free solution, prepared as above, add 20 ml. of *neutral formaldehyde solution* and titrate with *0.2 N sodium hydroxide solution* until the color matches that of the control solution, then add a few milliliters more and titrate back with *0.2 N hydrochloric acid* until the color is less pronounced than that of the control solution. Finally complete the titration with the *standard alkali* until the colors match perfectly.

CALCULATION. Subtract from the number of milliliters of alkali the number of milliliters of acid and multiply by 2.8, thus obtaining the number of milligrams of nitrogen as neutral amino acids in the 80-ml. aliquot employed. To obtain the amount in the sample weighed for analysis multiply by 1.25.

Van Slyke Nitrous Acid Gasometric Method.[25] This method employs nitrous acid which spontaneously decomposes with the formation of nitric oxide, displacing all air from the apparatus and, after the introduction of the amino solution, causes the evolution of nitrogen mixed with nitric oxide, the oxide being absorbed by alkaline permanganate solution and the nitrogen measured in a special buret.

APPARATUS. The *Assembly* is shown in Fig. 46 and details, other than the water or electric motor and the Hempel pipet, in Fig. 47.

REAGENTS. *Alkaline Permanganate Solution.* Dissolve 50 g. of $KMnO_4$ and either 25 g. of KOH or 18 g. of NaOH in water and dilute to 1 liter.

Caprylic Acid.

PROCESS. Ignore very small quantities of ammonia in the material, as Van Slyke's experiments show that only 21.6, 36.3, and 62.1% of the ammonia nitrogen is evolved in 3, 5, and 10 minutes respectively. Remove considerable quantities of ammonia by distillation under diminished pressure with *calcium hydroxide*, neutralizing the excess with *acetic acid* after the distillation.

Addition of Reagents. Fill *F* with water and from it the capillary leading to the Hempel pipet and the other capillary as far as *c*. Into *A* pour *glacial acetic acid* up to the mark, the quantity being sufficient to fill

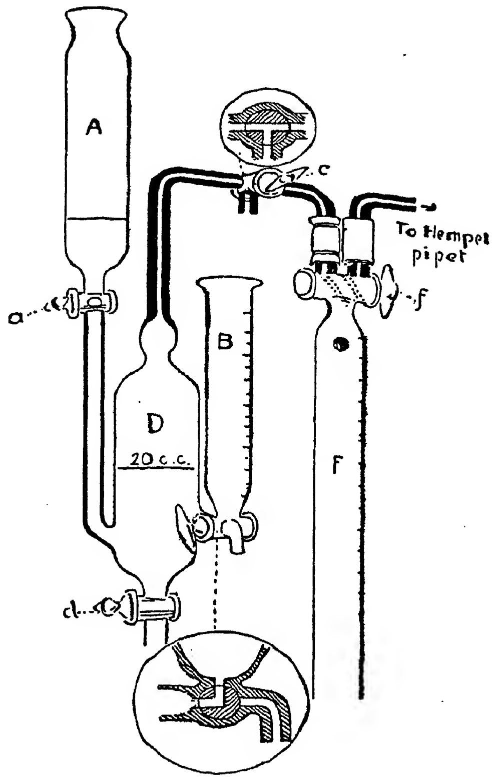
one-fifth of D into which it is run by opening the stopcock a, the air being allowed to escape through c. Into A pour sufficient *30% sodium nitrite solution* to fill D and the tube of A up to the mark a little above the cock. Close c, keep a open, and shake D for a few seconds until all of the solution but 20 ml., as shown by a mark, has been dispelled by nitric oxide and driven back into A. Close a, open c, connect D with the motor and shake for 2 minutes to expel the last of the air. Turn c and f so that D and F are connected.

Addition of Amino Solution. Add to the tube B 10 ml. or, if that amount contains more than 20 mg., a smaller amount of the solution of amino acids ready-formed or obtained by hydrolysis (see subsequent method), removing any excess through the overflow tube, and run into D. In operating with meat extracts, α-amino acids, and proteins, as well as partially or completely hydrolized proteins, shake D with the motor vigorously for 5 minutes. This completes the reaction except for 5% of lysine nitrogen which is a negligible proportion of the total nitrogen of a complete protein. To determine ammonia, methylamine, amino purines, and usually amines other than α-amino acids, add the reagents, allow to stand 1 to 2 hours, then shake 2 or 3 minutes.

The foaming over into F of a viscous solution may be prevented by rinsing out B and adding through it a little *caprylic alcohol* into D or, better, adding the caprylic alcohol with the glacial acetic acid before the amino solution.

Alkaline Permanganate Treatment. After the reaction is complete, open a, drive all the gas from D into F by liquid from A, and then, by raising the leveling bulb, from F into the Hempel absorption pipet containing *alkaline permanganate solution.* Slowly shake the latter by the motor for 2 minutes; this, except for a nearly exhausted solution, completes the absorption of nitric oxide.

Reading. Run the pure nitrogen back into F, raise the leveling bulb so that the surface of the water is on the level with the meniscus and read the volume of gas in F, the temperature, and barometric pressure. During



Courtesy of the Author, the J. Biol. Chem., and the Methods of Analysis of the A.O.A.C.

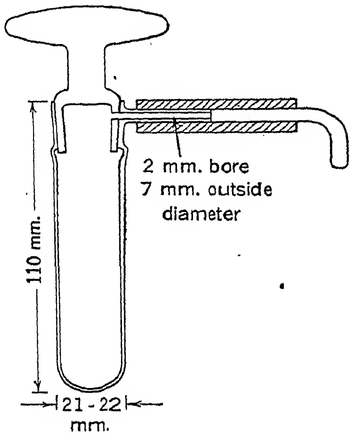
47. Van Slyke Deaminizing Bulb and Connection.

these operations leave a open to permit displacement of liquid as nitric oxide forms in D.

Check. To test the completeness of the reaction, drive out the nitrogen from F through c, close a, and connect D with F, shake out and drive over into F the gas formed in D

during the preceding operations, and from *F* into the Hempel pipet as before. After absorbing the nitric oxide, no more gas should remain than in blank tests—usually less than 0.1 ml.

After forcing all the gas from *D* into *F*, run the nitrous solution out through *c*, rinse *B*, and dry with a roll of filter paper or with *ethanol* and *ether*.



Courtesy of the Authors and J. Biol. Chem. 1941, **141,** *634*

FIG. 48. Van Slyke, Dillon, MacFadyen, and Hamilton Reaction Vessel.

Blank. Make on every fresh lot of nitrite blank determinations as described, except that 10 ml. of distilled water replace the amino solution. Nitrite giving a larger correction than 0.3 to 0.4 ml. in a 5-minute blank should be rejected.

Precaution Against Nitric Oxide Poisoning. Considerable amounts of nitric oxide are evolved from *A*. Either operate the apparatus in a hood or hang over *A* an inverted funnel connected with suction to draw off the oxide fumes.

CALCULATION. Calculate the percentage of nitrogen of amino acids by the formulas given for the Schulze Modification of the Schloesing Nitric Oxide Gasometric Method below, substituting in the second formula 0.001251 for 0.0006256 in obtaining the value for *G*, since the molecule of nitrogen gas contains two atoms of nitrogen, whereas the molecule of nitric oxide contains but one.

Van Slyke, Dillon, MacFadyen, and Hamilton Carboxyl Group Ninhydrin Gasometric Method.[26] Each known amino acid liberated from proteins by hydrolysis, when boiled with an excess of ninhydrin (triketo-hydrindene hydrate), evolves 1 mole of CO_2 from the carboxyl group, with the exception of aspartic acid and cystine which yield 2 moles. The reaction was discovered by Ruhemann[27] and is specific for free amino acids, since it requires the presence of the unconjugated carboxyl and α-NH_2 or (in proline and hydroxyproline) α-$NH(CH)_2$ groups.

APPARATUS. *Van Slyke-Neill Manometric Assembly* (Fig. 53).[28]

Pyrex Reaction Vessel (Fig. 48). The form shown in the cut is that most used. Vessels exposing the gases to rubber during the heating with ninhydrin are to be avoided, as rubber takes up and gives off carbon dioxide.

Four Glass Measuring Spoons, calibrated to hold 50 and 100 mg. of ninhydrin and 50 and 100 mg. of citrate buffer.

Water Bath. A cylindrical sheet metal vessel of a capacity to permit heating to 99 to 100° in 2 minutes and holding at not less than 98° when the tubes are immersed.

REAGENTS. *Ninhydrin* (Eastman Kodak Company or made from ethyl phthalate).[29] Grind to a fine powder and store in glass.

Citrate Buffers. (*A*) pH 4.7: Weigh 17.65 g. of trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and 8.40 g. of citric acid ($C_6H_8O_7 \cdot H_2O$). (*B*) pH 2.5: Weigh 2.06 g. of trisodium citrate and 19.15 g. of citric acid. Grind the ingredients of each to a fine powder, mix, then grind again. Both should yield no carbon dioxide in a blank analysis.

Lubricant for Stopper of Reaction Vessel. A lubricant which will not change its vis-

cosity much when heated from room temperature to 100° is necessary. It can be made by the formula of Puddington [30] as follows. Mix 35 g. of aluminum distearate to a paste in 100 ml. of heavy paraffin oil. Heat and stir over low flame for 1 or 2 minutes till a clear jelly forms. Cool and work up to a translucent paste on a glass plate with a steel spatula. (A lubricant with the same properties can be purchased under the name Nevastane XX from the Keystone Lubricating Co. or from E. Machlett and Son, New York.)

Alkali-Hydrazine Solution. See Van Slyke and Folch (Wet Combustion Manometric Method for carbon, above). Two milliliters of the solution should yield only enough carbon dioxide to exert about 8 mm. pressure at 2 ml. in blank analyses, equivalent to a carbon dioxide concentration of 0.0004*M*.

Sodium Hydroxide Solution, about 5 *N*. Dilute 1 volume of the 1 + 1 solution with 3 volumes of water.

Lactic Acid Solution, about 2 *N*. Dilute 2 volumes of concentrated lactic acid (sp.gr. 1.20) to 10 volumes with water.

Phosphoric Acid, 6 *M*. Mix sirupy phosphoric acid (sp.gr. 1.72) with 1.5 volumes of water. Dilute a suitable volume with 100 parts of water and titrate with 0.1 *N* alkali to the full red color of phenolphthalein. At 100 dilution the titration should show 0.12 *N*. Adjust the stock solution to exactly 6 ($\pm$0.1)*M*. The solution is for use when the ninhydrin reaction is run at approximately *p*H 1, 0.2 ml. of the 6 *M* acid added to 1 ml. of water forming 1 *M* phosphoric acid with a *p*H slightly under 1.

PROCESS. A. SUBMICRO AND MICRO ANALYSIS. For minimal amounts of amino acids, use the submicro technique; for most work the micro technique.

Range. For submicro analysis, measuring the P_{CO_2} at 0.5 ml., the following range to give a P_{CO_2} of 100 to 500 mm. applies: carboxyl carbon 0.035 to 0.18 mg., carboxyl nitrogen 0.04 to 0.21 mg.; for micro analysis (P_{CO_2} 2.0 ml.), weigh 0.14 to 0.7 and 0.2 to 0.8 respectively.

Carboxyl nitrogen (COOH—N), calculated as carboxyl carbon $\times$ 14/12, indicates α-nitrogen values estimated as 1 atom of nitrogen per molecule of carboxyl CO_2 evolved in the ninhydrin-CO_2 analysis. This is valid for all amino acids but aspartic.

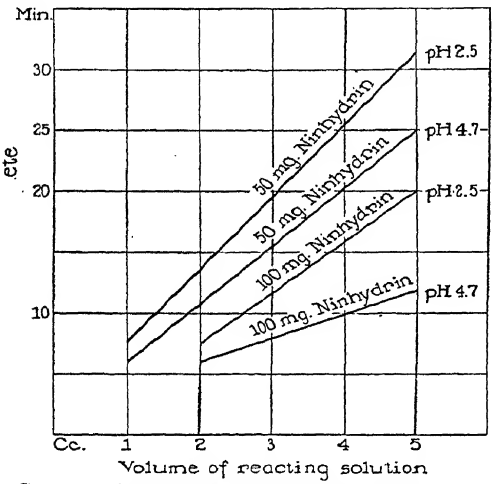
Charge. Place 4 to 8 mg. of the dry amino acid, weighed on a counterbalanced scoop (see Van Slyke and Folch Wet Combustion Manometric Method for carbon above), or 1 to 5 ml. of a solution, in one of the reaction vessels (Fig. 48).

Adjustment of pH. For general biological material, *p*H 2.5 is suitable, the yield being theoretical for amino acids except glycine, tryptophan, and cystine, which are low (95, 90, and 95% respectively) and high for lysine (105%). If, however, histidine, arginine, lysine, hydroxylysine, and cystine are precipitated with *phosphotungstic acid,* theoretical results are obtained at *p*H 1 and 8 to 9 minutes of boiling with 42 mg. of *ninhydrin* per milliliter of reacting solution, whereas the monoamino group of the filtrate requires *p*H 4.7, the only exception being the 2 to 3% excess yielded by glutamic and hydroxyglutamic acids. If no significant amounts of free acid or alkali or buffers other than the amino acids themselves are present, add for *p*H 2.5 to 4.7 50 mg. of *citrate buffer* for 1 to 2 ml. of sample, 100 mg. for 3 to 5 ml. If significant amounts of buffer or free acid or alkali are present in the unknown, add a drop of *bromophenol blue* or other indicator changing color at *p*H 3 to 4, and bring just to acidity, then add *citrate buffer* or *phosphoric acid.* Usually this preliminary adjustment is not necessary.

Removal of Preformed Carbon Dioxide. Add a few bits of *alundum* as anti-bump, and a drop of *caprylic acid* as anti-foam, then boil vigorously over a small flame for 20 to 30 seconds. If α-keto acids or other

substances yielding CO_2 at 100° are possibly present, continue the heating or boiling until the carbon dioxide is completely evolved, then stopper while hot, if the solution measures more than 2 ml., and keep closed until the ninhydrin is added, thus excluding atmospheric carbon dioxide.

Chilling. Cool to below 20, 25, or 25°, if the *p*H is 4.7, 2.5, or 1 respectively. If the



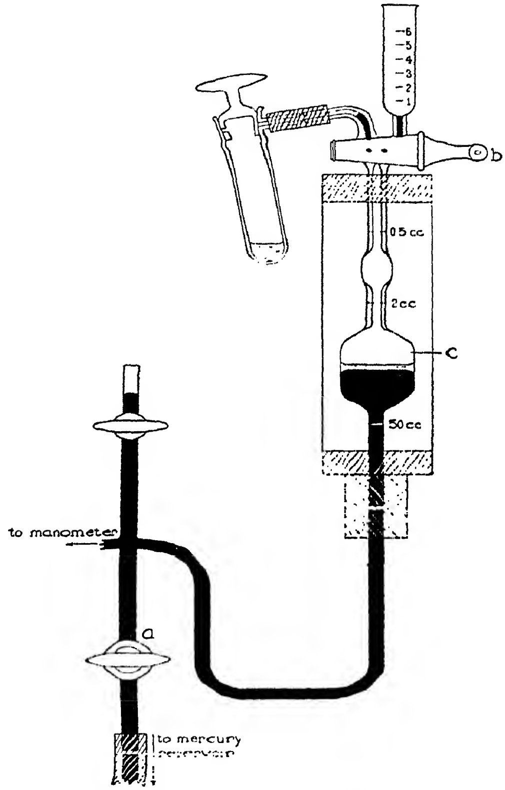
Courtesy of the Authors and J. Biol. Chem. 1941, **141**, *643*

FIG. 49. Van Slyke, Dillon, MacFadyen, and Hamilton Graph.

ninhydrin addition were made to a warmer solution, some amino acid carboxyl CO_2 might be evolved and lost before the subsequent evacuation of the vessel could be completed. If cooled to 10°, several minutes are allowable between ninhydrin addition and evacuation.

Ninhydrin Addition. Add to the chilled solution from a glass spoon 50 mg. (for 1 or 2 ml.) or 100 mg. (for 3 or 5 ml.) of *ninhydrin.*

Evacuation. Immediately place the lubricated stopper of the reaction vessel (Fig. 48) in position, with the side outlet open, and connect the outlet tube to a suction pump. Evacuate as quickly as possible to 20 or 30 mm. pressure. With a good water pump this requires only about 10 seconds. While the pump is still in action close the



Courtesy of the Author and J. Biol. Chem. 1941,

FIG. 50. Van Slyke Manometric Chamber with Reaction Vessel Attached but not yet Connected with Interior of Chamber.

clamp on the short rubber tube which covers the side tube of the vessel, then disconnect the pump.

Heating. Completely immerse the reacting vessel in the actively boiling water and hold in the bath for a period depending on the ninhydrin concentration and the *p*H as shown by Fig. 49, shaking gently while

in the bath after the first minute. Lysine, cystine, and glutamic acid give more than theoretical yield if the heating is unduly prolonged, whereas peptides and proteins are very slowly hydrolyzed.

Absorption of Carbon Dioxide. Place in the Van Slyke and Neill chamber 2 ml. of the *0.5 N sodium hydroxide plus hydrazine solution*, as directed by Van Slyke and Folch in their Wet Combustion Manometric Method for carbon determination (Part I, C1*b*). *Bring the reaction vessel to 38 to 40°*, attach to the manometer chamber, and lower the mercury to the middle of the chamber (*C*), as shown in Fig. 50, then complete the connection by opening the stopcock (*b*) and turning the stopper of the reaction vessel. Transfer the carbon dioxide from the warm reaction vessel to the alkali in the chamber by raising or lowering the mercury, making each of 6 to 10 round trips for 1 to 5 ml. of solution respectively in about 10 seconds. *Shake the reaction vessel with a swirling motion during each lowering of the reaction vessel.* After the last upward trip, lower the mercury to the middle of the chamber, close both the cock at the top and that leading to the leveling bulb, then remove the reaction vessel and seal the capillary with mercury from a small bottle into which dips a short capillary tube connected to *b* by a rubber connection (Van Slyke and Folch).

Manometric Determination of Carbon Dioxide. The technique is that described by Van Slyke and Folch. Take the manometric reading with the gas volume at 0.5 or 2.0 ml., depending on the amount of amino acid carboxyl as indicated under Range above.

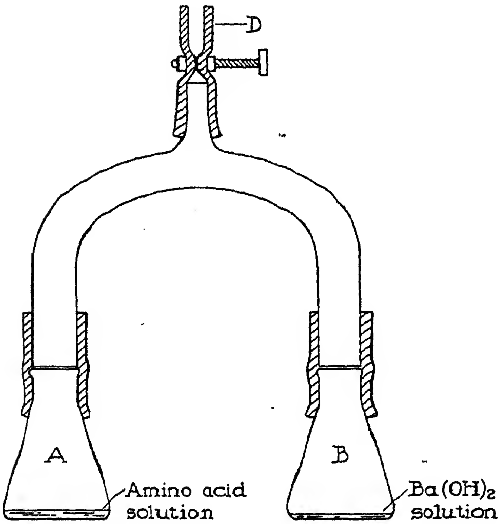
Blank. Obtain the correction (*c*), which is the value $p_1 - p_2$ found in a blank analysis, using all the reagents (except ninhydrin which does not contribute to the correction) but no amino acid. Redetermine the correction, which is usually 6 to 7 mm. measured at 2 ml. volume or 25 to 30 mm. at 0.5 ml. volume, for each fresh lot of 0.5 *N* sodium hydroxide solution.

FACTORS FOR CARBOXYL NITROGEN CALCULATION (VAN SLYKE, DILLON, MACFADYEN, AND HAMILTON)

Temperature	Factors *		
	Submicro analysis a = 0.5 ml.	Micro analysis a = 2.0 ml.	Macro analysis a = 10 ml.
°C.			
10	0.0004387	0.001720	0.008519
11	365	710	474
12	342	701	432
13	321	692	390
14	300	684	349
15	279	676	309
16	259	668	269
17	238	661	230
18	218	653	190
19	197	645	151
20	178	637	113
21	158	630	076
22	139	622	039
23	120	615	002
24	102	608	0.007967
25	084	601	931
26	066	594	897
27	049	587	863
28	032	580	830
29	015	573	797
30	0.0003998	567	765
31	982	560	732
32	966	554	700
33	950	548	670
34	934	541	640
35	918	536	609

* a indicates the gas volume at which the CO_2 pressure is measured.

B. MACRO ANALYSIS. For unknowns with 1.8 to 4.0 mg. of carboxyl nitrogen, proceed as with smaller samples except: (1) use a minimum volume of 2 instead of 1 ml., (2) increase the amount of *ninhydrin* to 150 mg. and of *citrate buffer* to 200 mg., (3) measure the carbon dioxide pressure with the gas at



Courtesy of the Authors and J. Biol. Chem. 1941, **141**, *675*

FIG. 51. Van Slyke, MacFadyen, and Hamilton Evolution and Distillation Assembly.

10 ml. volume, and (4) add a drop of *caprylic alcohol* as an anti-foam.

C. ALTERNATE PROCEDURE USING CARBON COMBUSTION ASSEMBLY. This is the earlier method of Van Slyke and Dillon [31] which is only about half as rapid as the above and more subject to error from atmospheric CO_2. The combustion tube (Fig. 31, *B*) is used as the reaction vessel and the heating is by a micro burner with the vessel attached to the chamber, as in the combustion. The procedure enables the combustion tube to be used also as a reaction vessel for the ninhydrin method.

D. CALCULATION. Obtain the milligrams of *carboxyl carbon* by the carbon factors corresponding to those used in the Van Slyke and Folch Wet Combustion Manometric Method for carbon above (Part I, C1*b*). To calculate *carboxyl nitrogen*, use the same factors multiplied by 14/12.

Van Slyke, MacFadyen, and Hamilton Carboxyl Group Ninhydrin Volumetric Method.[32] It is stated that this method has certain advantages over the previously published Christensen, West, and Dimick Method [33] in which the principles developed by Van Slyke and Dillon [34] were applied. The CO_2, evolved by the Van Slyke, Dillon, MacFadyen, and Hamilton Method above, is distilled into barium hydroxide solution and the excess of the latter is titrated. The method cannot be applied if volatile organic acids are present, as they would be distilled and titrated with the CO_2.

APPARATUS. *Evolution and Distillation Assembly* (Fig. 51). The U-tube and the necks of the 25-ml. Erlenmeyer flasks are 16 to 17 mm. in diameter.

Buret, 5 ml., accurate to 0.01 ml.

Rehberg Micro Buret, 200 c.mm.

Carbon Dioxide-Free Air Reservoir, consisting of two 2-liter aspirator bottles, their lower openings connected by a rubber tube, charged with 2.5 liters of 10% sodium hydroxide solution. The upper opening of one bottle is provided with a soda lime tube, the other with a perforated stopper with a rubber tube ending in a glass capillary. When all the air from the latter bottle has been used, interchange the soda lime tubes and air exit tube.

REAGENTS. *Citrate Buffers* and *Ninhydrin.* See Van Slyke, Dillon, MacFadyen, and Hamilton Method above.

Barium Hydroxide Reagent, about 0.25 *N*, for macro analyses. Titrate a saturated solution and dilute to 0.3 *N*, then mix 5 volumes with 1 volume of neutral 12% $2H_2O$ solution.

Barium Hydroxide Reagent, about 0.125 N, for micro analyses. Mix 5 volumes of a 0.15 N solution with 1 volume of 12% $BaCl_2 \cdot 2H_2O$ solution.

Barium Hydroxide Reagent, about 0.0155 N, for submicro analyses. Mix 1 volume of 0.125 N barium hydroxide solution with 7 volumes of 12% neutral barium chloride solution.

Standard Hydrochloric Acid, 0.1428, 0.07138, and 0.02855 N, equivalent to 1, 0.5, and 0.2 mg. of carboxyl nitrogen per milliliter.

Octyl Alcohol.

Indicators. Phenolphthalein, 1% in ethanol, and *cresol red,* 0.04% in water.

Sodium Veronal Buffer, of pH 8.0.[35] Mix 7 ml. of a 20.6% stock solution of sodium veronal with 4 ml. of 0.07143 N HCl.

PROCESS. *Ninhydrin Reaction.* Place the amino acid solution (not over 2 ml. for submicro, but as high as 5 ml. for micro or macro analyses) in flask A (Fig. 51). Add buffer to pH 2.5 or 4.7 (see preceding method) and a drop of *octyl alcohol,* then boil off preformed carbon dioxide, stopper the flask, and cool the solution to below 15°. Pass 100 ml. of *carbon dioxide-free air* through B and continue the stream up to 250 ml. while pipeting into the flask for macro, micro, and submicro analysis 3 ml. respectively of *0.25 N,* 1 ml. of *0.125 N,* and 1 ml. of *0.0155 N barium hydroxide reagent.* To the amino acid solution in flask A, add from a glass spoon 50, 100, or 150 mg. of *ninhydrin* (see preceding method), quickly connect with the U-tube (Fig. 51), the lower ends of the rubber tubes having been dipped in water for lubrication, exhaust immediately, and close the clamp at the top.

Immerse the apparatus up to the clamp in boiling water, as in the preceding method, for 6 and 7 minutes respectively at pH 4.7 and 2.5 if the ninhydrin concentration is 50 mg. per ml., and longer if the solution is more dilute.

Carbon Dioxide Distillation. Most of the material passes over on simply lifting B over the edge of the hot water bath (shaking B is essential for complete absorption) and immersing the lower half of B in cold water; A and the limb of the U-tube above A remain in the boiling water. The distillation consumes 2 minutes for 1 to 2 ml. and 3 minutes for 3 to 5 ml. of the amino acid solution. After cooling, admit *carbon dioxide-free air* from the reservoir through D, then close the clamp.

Titration. For macro and micro analyses, disconnect the flask and titrate at once with respectively *standard 0.1428 and 0.02855 N hydrochloric acid* from 5-ml. burets, using 1 drop of *phenolphthalein indicator.* For submicro analyses, titrate with *standard 0.07138 N hydrochloric acid* from a Rehberg micro buret with submerged tip, using 1 drop of *cresol red indicator,* and match against an equal volume of *veronal buffer* and *indicator.* During the titration, pass *carbon dioxide-free air* through the solution rapidly, but without splashing.

Blank. With each series, except macro analyses, make blank determinations, using water instead of the amino acid solution, and the same amount of reagents as for the unknown except ninhydrin. Use the volume (T_1) of standard acid required in the blank to neutralize the barium hydroxide after the distillation as a basis for the calculation. Ignore traces of carbon dioxide yielded by water and reagents in the blank, since these are covered by T_1.

CALCULATION. Obtain the milligrams of carboxyl nitrogen or carbon (M) by the formula

$$M = (T_1 - T_2) \times F$$

in which T_1 and T_2 are the milliliters of 0.1428, 0.07138, or 0.02855 N acid used in the titration of the blank and the unknown respectively, and F is the factor for the three normalities, namely 1, 0.2, and 0.5. The

corresponding factors for carboxyl carbon are 0.857, 0.1715, and 0.4285.

Individual Amino Acids

Classification. Vickery and Schmidt [36] list twenty-one amino acids which have been definitely isolated as components of protein molecules. At the date of writing at least another (hydroxylysine) should be added. The following classification is chemical, whereas that of the descriptions of the methods is alphabetical for greater convenience.

I. Aliphatic amino acids.

A. Monoamino-monocarboxylic acids.

1. Glycine (glycoll) or amino-acetic acid.

$$CH_2(NH_2)\cdot COOH$$

2. *d*-Alanine or α-amino-propionic acid.

$$CH_3\cdot CH(NH_2)\cdot COOH$$

3. *l*-Serine or β-hydroxy-α-amino-propionic acid.

$$CH_2(OH)\cdot CH(NH_2)\cdot COOH$$

4. *d*-Threonine or α-amino-β-hydroxy-butyric acid.

$$CH_3\cdot CH(OH)\cdot CH(NH_2)\cdot COOH$$

5. *d*-Valine or α-amino-iso-valerianic acid.

$$(CH_3)_2CH\cdot CH(NH_2)\cdot COOH$$

6. *l*-Leucine or α-amino-isocaproic acid.

$$(CH_3)_2CH\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

7. *d*-Isoleucine or α-amino-β-methyl-β-ethyl-propionic acid.

$$CH_3(C_2H_5)\cdot CH\cdot CH(NH_2)\cdot COOH$$

B. Monoamino-dicarboxylic acids.

8. *l*-Aspartic acid or amino-succinic acid.

$$HOOC\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

9. *d*-Glutamic acid or α-amino-glutaric acid.

$$HOOC\cdot (CH_2)_2\cdot CH(NH_2)\cdot COOH$$

10. *d*-Hydroxyglutamic acid or α-amino-β-hydroxyglutaric acid.

$$HOOC\cdot CH_2\cdot CHOH\cdot CH(NH_2)\cdot COOH$$

C. Diamino-monocarboxylic acids.

11. *d*-Arginine or α-amino-δ-guanidine-valeric acid.

$$NH_2\cdot CNH\cdot NH\cdot (CH_2)_3\cdot CH(NH_2)\cdot COOH$$

12. *d*-Lysine or α-ϵ-diamino-caproic acid.

$$)\cdot COOH$$

D. Sulfur-containing amino acids.

13. *l*-Cystine or dicysteine or di-β-thio-α-amino-propionic acid.

$$HOOC\cdot CH(NH_2)\cdot CH_2\cdot$$
$$S\cdot S\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

14. *l*-Methionine or α-amino-γ-methylthiol-*n*-butyric acid.

$$CH_3\cdot S\cdot (CH_2)_2\cdot CH(NH_2)\cdot COOH$$

II. Aromatic amino acids.

15. *l*-Phenylalanine or α-amino-β-phenyl-propionic acid.

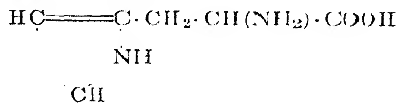
$$(C_6H_5)\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

16. *l*-Tyrosine or β-parahydroxy-phenyl-α-amino-propionic acid.

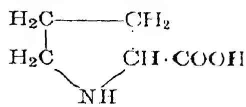
$$C_6H_4(OH)\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

III. Heterocyclic amino acids.

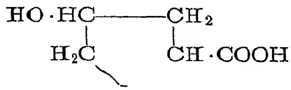
17. *l*-Histidine or β-imidazole-α-amino-propionic acid.



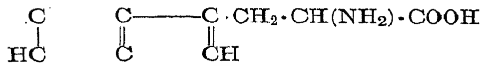
18. *l*-Proline or α-pyrrolidine-carboxylic acid.



19. *l*-Hydroxyproline or γ-hydroxy-α-pyrrolidine-carboxylic acid.



20. *l*-Tryptophan or β-indole-α-amino-propionic acid.



IV. Aromatic iodine-containing amino acids.

21. Iodogorgoic acid or 3,5-diiodo-tyrosine.

$$C_6H_2I_2(OH) \cdot CH_2 \cdot CH(NH_2) \cdot COOH$$

22. Thyroxin or β-[3,5-diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy) phenyl]-alanine.

$$C_6H_2I_2(OH) \cdot O \cdot C_6H_2I_2 \cdot CH_2CH(NH_2) \cdot$$

Fischer Comprehensive Ester-Isolation Method.[37] Although the percentage composition of proteins, in terms of constituent amino acids as given in standard works, was largely obtained by protein chemists using Emil Fischer's laborious ester-isolation method, details of the method would be out of place in this work, since the operations are such as can be satisfactorily carried out only in organic research laboratories.

Kossel and Kutscher Comprehensive Method.[38] Although limited to the basic amino acids and in part superseded by later methods, the Kossel and Kutscher method involves certain fundamental reactions which have not lost in importance through the years.

Process. *Hydrolysis.* Reflux 25 to 50 g. of the protein for 14 hours with a mixture of 3 parts by weight of *sulfuric acid* and 6 parts of water. Cool and make up to 1 liter in a volumetric flask.

In an aliquot of 5 to 10 ml. determine nitrogen by the Kjeldahl method and calculate the percentage of protein. Also determine the nitrogen content of the cork, if this is not already known.

First Precipitation with Barium Hydroxide. Mix the remainder of the acid solution with sufficient hot *concentrated barium hydroxide solution* to nearly precipitate all the sulfuric acid, leaving the reaction weakly acid. Filter on a Büchner funnel, boil the precipitate with three consecutive portions of water, filtering after each, then wash further with hot water. Evaporate the filtrate and washings and make up the combined solutions to 1 liter in a volumetric flask. Use aliquots of this "baryta filtrate" for subsequent determinations.

A. Ammonia Nitrogen. In duplicate aliquots of 100 ml. of the "baryta filtrate" determine nitrogen as ammonia by distillation with *magnesium oxide* and titration of the distillate.

B. Humin Nitrogen. In a 5- to 10-ml. aliquot of the "baryta filtrate" determine nitrogen, thus obtaining the amount remaining in the barium sulfate precipitate.

Second Precipitation with Barium Hydroxide. Add *magnesium oxide* to the remainder of the "baryta filtrate" and heat in a large porcelain dish until all the ammonia is removed, then add the duplicate solutions remaining after the distillation for the determination of nitrogen as ammonia. Add to the combined solutions *barium hydroxide solution* to strong alkaline reaction, filter, and boil the precipitate as before with three portions of water, and wash in addition with hot water.

Removal of Excess of Barium. To the filtrate and washings, add *sulfuric acid* to acid reaction, filter, and wash the barium sulfate thoroughly with hot water. Evaporate the filtrate and washings to a volume of less than 1 liter and make up to that volume in a volumetric flask.

Determine the nitrogen by the Kjeldahl method in 5 or 10 ml. of the solution and calculate the humin nitrogen (second portion) remaining in the barium organic precipitate.

C. SEPARATION OF HISTIDINE AND ARGININE FROM LYSINE BY SILVER SULFATE. Transfer the acid filtrate from the foregoing precipitation of barium sulfate to a 5-liter flask, dilute to 3 liters, and heat on the water bath. To the hot liquid add finely ground *silver sulfate* in portions, with shaking and further heating, until in excess, as determined by the following test.

Transfer a few drops of the liquid by means of a glass rod to a watch-glass, containing *baryta water*, resting on a dark surface. If the precipitate thus formed is white, the amount of silver sulfate is insufficient or some remains undissolved. An excess of the silver salt is indicated by the formation of a yellow precipitate.

Saturation with Barium Oxide. When the excess of the reagent is assured, cool the liquid to about 40° and saturate it with *powdered barium oxide.* Filter on a Büchner funnel, remove the precipitate from the paper, grind in a mortar with *baryta water*, again filter, then wash thoroughly.

Use the precipitate for the determination of histidine and arginine, the filtrate ("silver-baryta filtrate") for the determination of lysine.

D. HISTIDINE. *Hydrogen Sulfide Precipitation.* Levigate the silver-baryta precipitate with water containing *sulfuric acid*, decompose with *hydrogen sulfide*, and collect the silver sulfide (contaminated with barium sulfate) on a filter. Remove the precipitate from the filter, boil with water, and again filter; then wash thoroughly with water. Evaporate the filtrate to remove the hydrogen sulfide and make up to 1 liter in a volumetric flask.

Determine nitrogen in 20 ml. of the liquid, thus obtaining the nitrogen in the silver and barium precipitates.

Barium Nitrate Precipitation. Neutralize the remainder of the liquid with *baryta water*, add *barium nitrate* as long as a precipitate is seen to form, filter, and wash.

Precipitation with Silver Nitrate. Evaporate the filtrate to about 300 ml. and add *silver nitrate solution* until a few drops give a yellow color with *baryta water*, when tested as directed above. Neutralize the solution accurately with *baryta water*, then, from a buret, add small portions of *baryta water* until the histidine silver is completely precipitated. This is shown by the failure of *ammoniacal silver solution*, added to a small portion removed by a glass rod, to produce a precipitate soluble in an excess of ammonia. When the precipitation is complete, filter, rub up the precipitate in a mortar, and wash thoroughly on the paper. Reserve the filtrate for the determination of arginine below.

Kossel and Patten [39] add sulfuric acid to 5% content, precipitate the histidine with mercuric sulfate, filter, wash with 5% sulfuric acid, and determine histidine in the precipitate and arginine in the filtrate. Mercuric sulfate also was found to separate histidine from aspartic acid in 2.5% sulfuric acid solution.

Histidine Silver Decomposition. Decompose the arginine-free silver precipitate with *hydrogen sulfide*, after rubbing up in a mortar with water acidulated with *sulfuric acid*, filter, and wash with boiling water. (If desired, make the filtrate up to 1 liter and determine nitrogen in 4 ml. to note the progress of the separation.) In any event, remove the sulfuric acid with *baryta water;* then remove the excess of the latter with *carbon dioxide* in the usual manner. Filter, evaporate the filtrate to dryness, take up in *10 to 20% silver nitrate solution* to which has been added 1 drop of *nitric acid*. Filter to remove a small amount of organic matter, wash with water, then precipitate the histidine by adding cautiously *dilute ammoniacal silver nitrate solution*, and filter. Decompose the

precipitate with *hydrochloric acid*, evaporate, and dry *in vacuo* at 40°, thus obtaining the histidine ($C_6H_9N_3O_2 \cdot 2HCl$) in the crystalline form for weighing.

E. ARGININE. *Barium Oxide Precipitation.* Saturate the filtrate from the histidine with *powdered barium oxide* and separate the precipitate by filtration on a Büchner funnel. Remove the precipitate from the filter, rub up with *baryta water*, then return to the paper and wash until the nitric acid is removed.

Decomposition with Hydrogen Sulfide. Again remove the precipitate from the filter, but this time rub up with *water containing sulfuric acid*, then decompose the precipitate with *hydrogen sulfide*. Filter, remove the precipitate of silver sulfide from the paper, boil with several successive portions of water, and wash with hot water. Evaporate the filtrate and washings and make up to 1 liter.

Determine nitrogen in a 10- or 20-ml. aliquot and calculate the amount of arginine.

Remove the sulfuric acid from the remainder of the solution by *baryta water*, and the excess of baryta from the filtrate by *carbon dioxide* in the usual manner.

Gravimetric Determination. For the determination of arginine as neutral nitrate, neutralize the solution with *nitric acid*, evaporate, dry at room temperature *in vacuo*, and weigh the residue ($C_6H_{14}N_4O_2 \cdot HNO_3 + \frac{1}{2}H_2O$).

Polarimetric Determination. Read in a tube of suitable length directly in angular degrees or convert from Ventzke degrees. According to Gulewitsch,[40] in a solution acidified with nitric acid, $[\alpha]_D = +25.48$; according to Leims, in a solution acidified with sulfuric acid it is +22.35. On evaporation of the former solution, the arginine may be obtained as a white crystalline mass with the formula $C_6H_{14}N_4O_2 \cdot 2HNO_3$ without ash or only a trace of magnesia.

Gulewitsch [41] corrects for solubility of the arginine-silver precipitate in 1 liter of the baryta-containing liquid by subtracting 0.036 g. from the weight of arginine found.

F. LYSINE. *Phosphotungstic Acid Precipitation.* Acidify the filtrate from the silver precipitate ("Silver-baryta filtrate") with *sulfuric acid*, precipitate the small amount of silver by *hydrogen sulfide*, filter, and boil the precipitate several times; then wash with hot water. (If desired, as a control, determine the nitrogen in an aliquot of the combined filtrate and washings and compare with the foregoing nitrogen determinations.) In any event, evaporate the liquid to 500 ml., add *sulfuric acid* to a final concentration of 5%, then *phosphotungstic acid* until, on further addition to a small filtered portion, the liquid remains clear 10 seconds. Filter on a Büchner funnel, rub up the precipitate with *5% sulfuric acid*, and wash thoroughly.

Determine nitrogen in the filtrate which contains the greater part of the monoamino acids; some, however, is lost in the phosphotungstic acid precipitate together with some nitrogen of unknown compounds that escaped the preceding precipitations.

Removal of Phosphotungstic Acid. Decompose the phosphotungstic acid precipitate with *barium oxide*, filter on a Büchner funnel, boil the precipitate with several portions of water, and wash with boiling water on the paper. Remove the excess of barium from the filtrate by *carbon dioxide* and evaporate the filtrate nearly to dryness. Take up the filtrate with water, remove any barium carbonate that separates by filtration, and again evaporate to small volume.

Picric Acid Precipitation. Add a small amount of *ethanolic picric acid*, together with *ethanol*, and stir. To the alcoholic solution add *picric acid* in small portions until no further precipitation is noted, avoiding an excess in which lysine picrate is soluble. After several hours, filter and wash with the least possible amount of *absolute ethanol*. Evapo-

rate the mother liquor and treat as directed below.

Dissolve the lysine picrate in boiling water, filter through a heated funnel, and evaporate to small volume, then allow to cool. Collect the needle-shaped crystals of lysine picrate which separate on a weighed paper, wash with a little *ethanol*, dry, and weigh. In the same way, treat the mother liquor and washing.

Calculation. Lawrow [42] corrects the weight of lysine for solubility in 100 parts of water by adding 0.54 g. Kossel and Kutscher, however, resort to the tedious task of working over the alcoholic and aqueous mother liquors, thus securing additional crops of crystals.

Glycine

Town Nitranilic Acid Gravimetric Method.[43] Town (St. Bartholomew's Hospital, London) employs as the chief reagent 2,5-dihydroxy-3,6-dinitro-*p*-benzoquinone, or nitranilic acid which acts as a strong dibasic acid. It forms a salt with glycine, with the formula $(C_2H_5O_2N)_2 \cdot C_6(OH)_2(NO_2)_2O_2$, that is soluble in water to 0.8%, but is practically insoluble in 80 to 100% ethanol. The reagent gives no precipitate with alanine, valine, leucine, phenylalanine, glutamic acid, or tryptophan, although the latter causes a strong darkening of the solution.

Reagent. *Nitranilic Acid.* Treat 20 g. of 1,4-diacetoxybenzene (obtained by acetylation of quinol) very slowly with about 10 ml. of fuming nitric acid (below 5°) and add the resulting solution also very slowly (allowing about 1 hour) to 70 ml. of *fuming nitric acid* at 2° with continuous mechanical stirring, keeping the temperature below 5°. Add to the liquid 65 ml. of H_2SO_4 at below 5° dropwise with stirring during about 3 hours. Continue the stirring for an additional hour, during which the nitranilic acid crystallizes out, then pour into 10 volumes of ice in a beaker. Cool in ice-salt and collect the crystals rapidly on a sintered glass funnel. Dry *in vacuo* over sulfuric acid and potassium hydroxide and recrystallize the yellow prisms (about 10 g.) from boiling ethyl acetate.

Process. *Hydrolysis.* Reflux 0.8 (caseinogen) to 8.0 (gelatin) g. of the protein with 25 (caseinogen) to 100 (gelatin) ml. of *hydrochloric acid* for 24 hours. Evaporate to dryness on a water bath to remove the excess of hydrochloric acid, dissolve the residue in water, filter to remove humin, and make up to the mark in a 25 (caseinogen) or 250 (gelatin) ml. flask.

Barium Hydroxide Treatment. To a 10-ml. aliquot, add *0.328 N barium hydroxide solution* (15 ml.) until distinctly alkaline to *phenolphthalein* and draw air through the solution for 1 hour, while heating at 70° in a water bath, to remove ammonia. Add *4 N sulfuric acid* (2 ml.) to acid reaction (*Congo red*), collect the precipitated barium sulfate on a filter, wash, and evaporate the combined filtrate and washings to 4 ml. Add to the liquid 30 ml. of *absolute ethanol* and remove the slight precipitate of barium sulfate by filtration.

Nitranilic Acid Precipitation. To the clear alcoholic solution, add 300 mg. of *nitranilic acid in 5 ml. of ethanol* and allow to stand overnight, although the crystalline precipitate forms almost immediately. Filter on a dry paper or Gooch crucible, wash with *absolute ethanol*, dry *in vacuo* over sulfuric acid for 24 hours, and weigh.

Zimmermann Phthaldialdehyde Colorimetric Method.[44] Zimmermann found that when a 1% solution of glycine is treated successively with 10 drops of *2 N sodium hydroxide solution*, 8 drops of *orthophthaldialdehyde*, and 10 drops of *hydrochloric acid*, a violet color is formed, whereas only a yellow-green color appears with ammonium salts and a yellow color with most of the amino acids of proteins. When applied to the phosphotungstic acid precipitate from hydrolyzates, the reaction was regarded as practically specific.

Zimmermann employed his reaction only as a qualitative test, but others have adapted it to quantitative determination.

I. Klein and Linser Modification.[45] In their studies Klein and Linser showed that, by varying the conditions, colors ranging through red, violet, blue, and green can be obtained by the Zimmermann method. The component colors, other than green, are formed by the action of the alkali on the amino acid and are soluble in dilute ethanol, whereas the green component is soluble in chloroform. By substituting phosphate buffer for the sodium hydroxide, only the green color is formed and this color serves for their colorimetric modification.

The same authors give instructions for the removal of tryptophan by precipitation with mercurous (*sic*) sulfate and ammonia by a suitable method, such as Folin's, employing weak alkali and low temperature.

APPARATUS. *Pulfrich Photometer. Micro Colorimeter.*

REAGENTS. *Phosphate Buffer, M/15, pH 8.0.*

Zimmermann Reagent. See Patton Modification below.

MACRO PROCESS (For 2 to 6.5 mg. of glycine). *Color Formation.* To 10 ml. of the solution, add 15 ml. of a mixture of 25 parts of *M/15 phosphate buffer* and 75 parts of the *Zimmermann reagent,* prepared immediately before use. Shake well and after exactly 2 minutes add 35 ml. of a mixture of 5 parts of *sulfuric acid* and 30 parts of *ethanol,* which also is prepared just before it is added to the solution. Again shake thoroughly, allow to stand 2 minutes, transfer to a separatory funnel, and add 30 ml. of *chloroform,* using all or a portion for rinsing. Shake well, allow to settle, and remove 5 ml. of the slightly turbid chloroform solution to the cell of the colorimeter. To remove the turbidity, add from a pipet 1 ml. of *ethanol.*

Color Reading. Determine the extinction coefficient in the Pulfrich photometer and compare with that of glycine solutions treated as in the actual analysis.

The extinction and concentration are proportional; 15 ml. of the reagent for 0.5 to 6.5 mg. of glycine are used, but, as noted below, with 2.0 mg. the green color is too faint for accurate estimation. When the concentration is higher than 6.5 dilute the colored solution or compare with a calculation curve plotted from data secured with the pure substance.

SEMI-MICRO PROCESS (For 0.15 to 2 mg. of glycine). *Color Formation.* Mix 0.5 ml. of the solution of the unknown with 0.75 ml. of the mixture of *buffer* and *reagent* as in the macro method. Allow to stand 2 minutes, then add 1 ml. of the *acid-ethanol mixture* and shake well. After 2 additional minutes, add 5 ml. of *chloroform* and shake.

Color Reading. When the layers separate, remove 3 ml. of the chloroform layer to the cell of the micro colorimeter, add 0.5 ml. of *ethanol,* mix, and read against a *standard solution of glycine* treated in like manner.

II. Patton Modification.[46] The details were developed at the University of Minnesota.

APPARATUS. *Colorimeter.*

REAGENT. *Zimmermann Reagent.*[47] Distil 2 g. of *o*-phthalaldehyde (Kahlbaum) with 300 ml. of water over a free flame or hot plate and save only the first 200 ml. Store in a brown bottle; it will keep several months.

If the chemical is not at hand, reflux 10 g. of ω-tetrabromo-*o*-oxylene and 9 g. of crystalline potassium oxalate with 62 ml. of water and 62 ml. of ethanol for 40 hours, then distil off 50 ml. of ethanol, add to the residual solution 10 g. of $Na_3PO_4 \cdot 12H_2O$ and 300 ml. of water and distil off 300 ml. of liquid. Store in brown bottles.

STANDARD. *Glycine Solution,* containing 1 g. of pure glycine in 1 ml.

PROCESS. *Hydrolysis.* Place 3 g. of the protein in a 300-ml. Kjeldahl flask, add 50 ml. of constantly boiling *hydrochloric acid,* and

gently reflux in an air bath over an electric plate. As soon as the protein is dissolved, add 1 ml. of *benzaldehyde* and boil 24 hours. Wash the mixture into a 1-liter Claisen flask and distil *in vacuo* in a water bath at 65° until only a paste remains, thus removing the benzaldehyde and most of the free hydrochloric acid.

Dilute the residue with 100 ml. of water, add a few drops of *butanol* to prevent foaming and an excess of *sodium bicarbonate*, then distil *in vacuo* the alkaline solution below 30 mm. at 40 to 50° to remove ammonia which interferes with the color reaction. When the volume of 20 ml. is reached and sodium chloride begins to precipitate, filter quickly, with suction, and wash the carbonate and chloride with a few milliliters of *70% ethanol*. Neutralize to *litmus paper* the filtrate with *hydrochloric acid* to *p*H 6 to 8, and again evaporate *in vacuo* to about 10 ml. Filter quickly on a small Witt plate, with suction, and wash the sodium chloride as before with a few milliliters of *70% ethanol*.

Dilute the filtrate to 100 ml. in a volumetric flask and determine nitrogen by the Kjeldahl method in an aliquot.

Color Formation. Pipet a 5-ml. aliquot of the solution into a 30-ml. test tube. Into another test tube, for a standard, pipet 5 ml. of a *zein hydrolyzate* and 1 ml. of *standard glycine solution* containing 1 mg. To the contents of both tubes add 2 ml. of *M/15 phosphate buffer* at *p*H 8.0, then immediately 5 ml. of the *Zimmermann reagent*. Mix thoroughly by shaking for 30 seconds and allow to stand 2 minutes, then add 5 ml. of a mixture of *60 ml. of ethanol* and 10 ml. of *sulfuric acid* freshly mixed and cooled. Mix, add 10 ml. of *chloroform*, stopper, shake vigorously for 30 seconds, and allow the chloroform layer to separate. Remove 5 ml. of the green chloroform solution to a colorimeter cup, add 1 ml. of *ethanol*, and stir with a small glass rod until all turbidity disappears.

Color Reading. Compare the known and unknown, treated in the same manner, in the colorimeter.

In subsequent tests, adjust the standard glycine solution at 20 mm. so that the unknown reads between 15 and 25 mm. A smaller amount of the sample than 3 g. may be used if the material is scanty.

CALCULATION. Obtain the grams of glycine (*G*) per 100 ml. of protein by the following formula:

$$C \qquad N \qquad \frac{A)}{} \times 100$$

in which *W* is the weight of glycine in the final hydrolyzate, *C* is the weight of protein taken, *T* is the total nitrogen in the protein taken, *H* is the humin nitrogen, *A* is the ammonia nitrogen, and *N* is the total nitrogen in the final hydrolyzate.

EXAMPLES. Zein 0, hordein 0, casein 0.50, edestin 1.60, arachin 2.05, gliadin 0.47, collagen 6.5, glutenin 0.81, glutelin (oat) 1.09, vitellin 0.81, and albumin 1.75%.

III. Brecht and Grundmann Modification.[48] The details up to the colorimetric reading differ little from those given by Patton. The authors note the importance of defatting with *ether* either before hydrolysis, which is preferable, or after.

Color Reading. Dilute 3 ml. of the chloroform layer with 0.5 ml. of *ethanol* and read the extinction coefficient in the Pulfrich photometer using filter S72. Compare the reading with the calibration curve. The extinction curve is linear only between 150 and 600 γ.

EXAMPLES. Bread 0.11, rice 0.16, potatoes 0.042, beef 0.66, fish muscle 0.16, egg albumin 0.27, and egg yolk 0.77%.

Rapoport Nitrite-Permanganate Volumetric Method.[49] The method (Vienna University) depends on the deamination of glycine by sodium nitrite into glycolic acid which in turn is oxidized to oxalic acid:

$$C_2H_4O_3 \qquad = C_2H_2O_4 + H_2O$$

PROCESS. *Hydrolysis.* Reflux 3 g. of the protein with 60 ml. of *20% hydrochloric acid* for 40 hours and drive off the hydrochloric acid *in vacuo*. Add *10% milk of lime suspension* to the residue until it is alkaline to *litmus*, then add 200 ml. of water and evaporate *in vacuo* to 10 ml. Dilute with 100 ml. of *ethanol* added in portions, filter through a pleated paper to remove the dicarboxylic acids, neutralize the filtrate with *acetic acid*, and evaporate the ethanol *in vacuo*. Dilute the residue to 250 ml. in a volumetric flask and remove an aliquot corresponding to 100 mg. of the protein to a 250-ml. Erlenmeyer flask.

Deaminization.[50] Adjust the aliquot of the hydrolyzate to 70 ml., add 0.5 ml. of *hydrochloric acid*, and heat in a boiling water bath. Add dropwise from a pipet 15 ml. of *2.5% sodium nitrite solution*, then, also from a pipet, in like manner 15 ml. of *7.5% urea solution*, allowing 20 minutes for the addition of each solution.

Oxidation. To the mixture add 1.5 g. of *sodium hydroxide* and concentrate to 4 to 8 ml. Add 15 ml. of *0.1 N potassium permanganate solution*, boil for 30 seconds, then add cautiously *sodium thiosulfate* equivalent to double that held on the point of a knife blade. After the somewhat violent reaction, heat again to boiling. As soon as the suspension clears up and finally becomes pure white, remove the flask from the heat, allow to cool somewhat, then add a few milliliters of *glacial acetic acid*. At this point the white precipitate dissolves and there is a strong evolution of hydrogen sulfide and sulfur dioxide. When entirely cool, add *strong ethanolic iodine solution* to a brown color, transfer to a large centrifuge tube, and add 4 ml. of *2 M calcium chloride solution*. Let stand 1 hour, centrifuge, wash with 2 portions of 10 to 15 ml. of water, mixing and centrifuging after each addition, and taking care that the supernatant liquid is clear each time. To the residue in the centrifuge tube add 5 ml. of *50% sulfuric acid* and heat for 3 minutes in a boiling water bath.

Titration. Deliver *0.1 or 0.05 N potassium permanganate solution* from a semi-micro buret in the usual manner.

CALCULATION. Use: 1 ml. of 0.1 N $KMnO_4$ = 0.5 ml. of 0.1 N glycolic acid = 3.80 mg. of glycolic acid = 3.75 mg. of glycine.

ALANINE

Kendall and Friedemann Nitrite-Lactic Acid Method.[51] Amino acids react with nitrous acid according to the following general reaction:

$$\cdot\ HNO_2 -$$

$$R\cdot CHOH\cdot COOH + \qquad + H_2O$$

Kendall and Friedemann employ the reaction for the determination of alanine by a method developed at Northwestern University. The lactic acid formed with the nitrous acid is determined by a method devised by the same authors.[52]

Certain interfering substances are removed by precipitation with cupric sulfate and calcium hydroxide.

APPARATUS. *Lactic Acid Assembly.*

PROCESS. *Reduction.* Place the requisite amount of the solution, containing a concentration of 10 to 50 mg. of alanine, in a 250-ml. volumetric flask and add water to about 75 ml., then add 0.5 g. of *sodium hydrogen sulfate* (0.25 ml. of hydrochloric acid may be substituted, but is less satisfactory). Bring to 93 to 95° in a boiling water bath. Introduce from a pipet, in such a manner that the capillary end rests on the bottom of the flask, 15 ml. of *2.5% sodium nitrite solution*, opening the pinchcock slowly to allow the nitrite to flow at a uniform rate of not more than 1 ml. per minute, with frequent shaking. Withdraw the pipet, rinsing back any adhering solution, then add 15 ml. of *7.5% urea solution* at the same rate and in the

same manner. Remove the flask from the water bath, wash down the neck with water, make up to 200 ml., and cool to room temperature (this may be expedited under the tap).

Lactic Acid Determination. If no interfering carbohydrates are present, make the volume up to 250 ml., shake, and aliquot for the lactic acid determination by the Friedemann and Kendall method.

To remove interfering substances add 10 ml. each of *20% cupric sulfate solution* and *20% calcium hydroxide suspension* before making up to 250 ml. Mix, allow to settle, filter into a dry flask, and use aliquots for the determination.

I. Fürth, Scholl, and Herrmann Micro Modification.[53] A number of defects of the original method have been rectified in this modification developed at Vienna University.

APPARATUS. *Embden and Lehnartz Oxidation Apparatus.*

Acetaldehyde Distillation Apparatus.

PROCESS. *Hydrolysis.* Reflux 10 g. of the protein with 200 ml. of *20% hydrochloric acid* for 48 hours and evaporate *in vacuo.* Add *10% milk of lime* to alkaline reaction, dilute to 200 ml., and expel the ammonia *in vacuo.* Filter and evaporate to about 10 ml.

Removal of Dicarboxylic Acids by Foreman Method. Transfer the solution to a 110 volumetric flask, rinse, and make up to the mark with *ethanol*, filter on a dry paper, and remove 100 ml. equivalent to $^{10}/_{11}$ of 10 g. (9.09 g.) of the sample for subsequent operations.

Phosphotungstic Acid Precipitation. Neutralize the filtrate from the ethanol precipitation with *acetic acid*, remove the ethanol by evaporation *in vacuo* to 50 ml., and precipitate with 15 g. of *phosphotungstic acid* with the addition of 18 g. of *hydrochloric acid* in a little water. Warm until the precipitate dissolves, then remove to a refrigerator where overnight the precipitate again forms. Filter into a 1-liter volumetric flask, wash until calcium salts are removed, and make up to the mark.

Treatment with Nitrite and Urea. Dilute two 1-ml. aliquots of the solution (= 10 mg. of the protein) to 75 ml., add 0.5 ml. of *hydrochloric acid*, and heat on a boiling water bath. Run in slowly in the course of 20 minutes 15 ml. of *2.5% sodium nitrite solution*, followed in like manner by 15 ml. of *7.5% urea solution* to remove the excess of nitrous acid from both aliquots, unite, and make up to 250 ml. in a volumetric flask. Each 10 ml. of the united solution corresponds to 0.8 mg. of the protein.

Permanganate Oxidation to Lactic Acid and Acetaldehyde. Treat aliquots of 5 to 20 ml. of the solution with *potassium permanganate solution* in the oxidation apparatus devised for the micro determination of lactic acid by Embden and Lehnartz.[54] The writers thus far have been unable to find the publication in the libraries. If details of the procedure are lacking, the method of Kendall and Friedemann may be substituted.

Acetaldehyde Separation. After the oxidation is complete remove the excess of sulfurous acid with *iodine* and decompose the acetaldehyde sulfurous acid with *solid disodium monohydrogen phosphate* into acetaldehyde and sulfurous acid, then add *0.005 N iodine solution* until *starch solution* produces a blue color. Acidify with *phosphoric acid* and transfer to flask *a*.

Acetaldehyde Distillation. Flask *a* is fitted with a double-bored stopper carrying a small tube (*b*) for the entrance of air and an upright condenser (*c*) of special efficiency in cooling. The delivery tube from *c* passes through one hole of a double-bored stopper of a small Erlenmeyer flask (*e*) containing 10 ml. of *0.02 N sulfurous acid solution.* Through the other hole passes the narrow tube of an adaptor (*f*) similar to a Gooch crucible holder, containing a sieve plate (*g*) and glass beads (*h*), and connected at the top

through a single-bored stopper with the exhaust.

Heat flask *a* in a water bath at 40° and continue the distillation 30 minutes, then disconnect the exhaust, wash the glass beads with water into flask *e* and carefully remove the excess of sulfurous acid with *iodine solution*. Decompose the acetaldehyde sulfite with *disodium phosphate* and titrate the bisulfite with *standard 0.005 N iodine solution*.

Examples. Silk fibroin 21.8, casein 5.3, zein 8.9, gelatin 2.5, and keratin 3.8% of alanine.

Note. McChesny[55] has shown that valine, leucine, and other interfering substances cause an error in the Kendall and Friedemann method requiring a correction as high as 9%.

II. Fromageot and Heitz Colorimetric Modification.[56] The addition of 1 volume of *5% mercuric acetate solution* to 10 volumes of the solution of the unknown after deamination of the amino acids inhibits the formation of acetaldehyde from serine and aspartic acid during the final oxidation by permanganate. The color subsequently formed with *sodium nitroprusside* and *piperazine* is specific for alanine, making possible the colorimetric determination of that amino acid in the presence of other aldehydes.

It is also possible, by omitting the mercury salt, increasing the manganic sulfate concentration, and extending the time for the reaction, to determine the sum of alanine, serine, and aspartic acid in the presence of other amino acids.

Accuracy within 5% of the truth is claimed for both operations.

Virtanen, Laine, and Toivonen Ninhydrin Iodometric Method.[57] Apparatus. *Friedemann et al. or Lieb and Zacherl Lactic Acid Apparatus.*

Process. *Acetaldehyde Formation and Distillation.* Place 10 ml. of a neutral solution of amino acids, containing 0.2 to 2.0 mg. of alanine, in the distillation flask of the lactic acid apparatus. To the solution add 7.5 g. of *ammonium sulfate* and 0.5 g. of *citric acid*, and heat to boiling in a slow current of air. Add 2 ml. of *1% ninhydrin (triketohydrindene) solution* and continue to boil for 30 minutes, collecting the acetaldehyde formed in 4 ml. of *1% sodium bisulfate solution.*

Acetaldehyde Titration. Proceed with the titration as directed for the Friedemann modification of the von Fürth and Charnass acetaldehyde distillation iodometric method, Part II, G1.

Note. The recovery ranges from 94 to 101%. Amino acids yielding other volatile aldehydes do not interfere.

To determine the sum of alanine, valine, leucine, isoleucine, phenylalanine, and methionine, heat with 1 g. of *monopotassium dihydrogen phosphate* and 1.5 g. of *sodium chloride* instead of ammonium sulfate and citric acid, then heat 15 minutes and steam distil 30 minutes. Results for alanine and for the sum of valine, leucine, and isoleucine were respectively as follows: zein 9.75 and 17.23, egg albumin 7.3 and 15.9, and casein 5.6 and 14.3% of nitrogen in the total nitrogen. For phenylalanine by the Kapeller-Adler method and methionine by the Baernstein method the results were respectively as follows: zein 4.00 and 1.32, egg albumin 2.9 and 2.8, and casein 2.7 and 2.0% of nitrogen in the total nitrogen. Dipeptides, glycine, serine, tyrosine, dihydroxyphenylalanine, tryptophan, proline, hydroxyproline, cystine, cysteine, arginine, histidine, lysine, and glutamic acid yield no volatile aldehydes. Aspartic acid yields 10% of acetaldehyde and β-alanine only a little. Methionine yields β-methylthiopropional.

Serine

Nicolet and Shinn Periodate Gravimetric Method.[58] As developed at the U. S. Department of Agriculture, formaldehyde,

formed by the action of periodic acid on serine, is precipitated as a derivative with dimedon (1,1-dimethyl-3,5-diketo-cyclohexane).

Apparatus. See Nicolet and Shinn Bisulfite Method for Threonine below.

Reagents. See Threonine below.

Process. *Hydrolysis.* Reflux for 24 hours 1 g. of the sample with an appropriate amount of *20% hydrochloric acid.* Remove the excess of acid by evaporation *in vacuo* and decolorize the hydrolyzate with a small amount of *norit,* which also reduces foaming.

Periodic Acid Treatment. See Threonine below.

In a single determination of serine alone, the tubes of *1.9% sodium meta-bisulfite,* used in the threonine method, obviously may be omitted, but threonine will often be determined simultaneously, and, if not, it will often be desirable to run two or more determinations in series, using the same gas stream. In such cases the *meta-bisulfite* is necessary and each pair of determinations should have a tube of *saturated sodium bicarbonate solution* inserted between them.

After removal of the acetaldehyde, transfer the solution to a 250-ml. Erlenmeyer flask, add 1 drop of *methyl red indicator* and then *acetic acid* dropwise until the color changes from yellow to faint red. At this stage the volume is usually about 50 ml.

Dimedon Precipitation. Precipitate the formaldehyde as the dimedon derivative by adding an excess of *0.4% dimedon solution* (at least double that required to react with all the aldehydes present) and allow the stoppered flask to stand 48 to 72 hours at room temperature. Both serine and threonine cause the formation of glyoxylic acid, much of which probably persists to this stage of the process.

Collect the precipitate on a sintered-glass Gooch crucible, wash, dry in a vacuum desiccator, and weigh. Test the purity of the precipitate by determination of the melting point (189°) and of the mixed melting point.

Calculation. Use: 1 mg. of dimedon derivative = 0.3596 mg. of serine.

Threonine

Shinn and Nicolet Bisulfite Volumetric Method.[59] In this method (U. S. Department of Agriculture) the acetaldehyde formed by the action of periodate on threonine is carried in a stream of carbon dioxide into a solution of sodium bisulfite which is then titrated iodometrically.

Apparatus. A train of three 2.5 x 20 cm. Pyrex test tubes arranged for gas absorption, except that tube 1 carries a dropping funnel, the stem of which extends nearly to the bottom, serving for the introduction of the periodate solution and the passage of carbon dioxide gas.

Reagents. *Sodium Arsenite Reagent,* 0.1 *N.* Dissolve 12.99 g. of $NaAsO_2$ in a solution of 20 g. of $NaHCO_3$ in 1 liter.

Sodium Meta-Bisulfite Solution, 1.9% Dissolve 19 g. of $Na_2S_2O_5$ in water and dilute to 1 liter.

Periodic Acid Solution. An approximately 0.5 *M* solution of H_5IO_6. (In the description of the procedure, the authors give 0.5 *N*.)

Process. *Introduction of Reagents.* Into tube 1 measure in the order named, (1) the solution of the sample, preferably containing 3 to 10 mg. of threonine in not much more than 5 ml. in volume, (2) 1 drop of *Nujol* to reduce foaming, (3) 5 ml. of *M sodium bicarbonate solution,* and (4) 10 ml. of *sodium arsenite reagent.*

Into tubes 2 and 3 respectively pipet 5 ml. and 3 ml. of *2% sodium meta-bisulfite solution,* each diluted to 25 ml.

Periodic Acid Treatment. Connect the dropping funnel with the *carbon dioxide* supply and run the gas through the train for several seconds to mix the contents. Break

the connection and introduce into the dropping funnel, with the stopcock closed, 1 to 2 ml. of *0.5 M periodic acid solution.* Reconnect the tubes, open the stopcock, and allow the *periodic acid* to enter under *carbon dioxide* pressure, thus guarding against loss of acetaldehyde and facilitating the introduction of the reagent against back pressure from the other tubes and contamination by acetaldehyde and acetone in the laboratory air. Continue the passage of *carbon dioxide* through the system for 1 hour at the rate of about 1 liter per minute.

Titration. When the absorption of the acetaldehyde by the sodium meta-bisulfite is complete, remove the contents of tubes 2 and 3 to a beaker and titrate as in the determination of lactic acid by the Clausen Acetaldehyde Modification of the von Fürth and Charnass Method, Part II, G1.

CALCULATION. Use: 1 ml. of 0.02 *N* iodine solution = 1.19 mg. of threonine.

Winnick Micro Diffusion Modification.[60] This modification (Wayne University) is characterized by its simplicity.

APPARATUS. *Conway Micro Diffusion Unit.*[61]

PROCESS. *Hydrolysis.* Reflux a weighed portion of the protein (usually about 0.5 g.) for 24 hours with 20 ml. of *3 N hydrochloric acid.* Cool, add *3 N sodium hydroxide solution* to pH 7.0, and dilute to 100 to 250 ml. in a volumetric flask.

Oxidation and Micro Diffusion. Pipet 1 to 1.5 ml. of *0.25 M sodium bisulfite solution* into the central chamber of the diffusion unit. Place in the outer chamber a 2- or 3-ml. aliquot of the neutralized hydrolyzate, then 1 ml. of *0.1 M tripotassium phosphate solution* and 1 ml. of *0.2 M periodic acid solution* to give a pH of 7.0. Adjust the cover, leaving a narrow slit through which to pipet 1 ml. of the *periodic acid solution* into the outer chamber. Immediately close the slit and rotate to mix the solutions. After 4 to 5 hours at room temperature, remove the cover and oxidize the excess of bisulfite with *1 N iodine solution.*

Titration. Add powdered *disodium phosphate* to dissociate the acetaldehyde–bisulfite compound and titrate the liberated bisulfite with *standard 0.005 N iodine solution*, following the details given under the Winnick Method for lactic acid (Part II, H1).

CALCULATION. Use: 1 ml. of 0.005 *N* iodine solution = 0.298 mg. of threonine.

EXAMPLES. Casein 4.40, 4.23, lactoglobin 5.25, 5.46, and gliadin 2.92% of threonine.

VALINE LEUCINE

Fromageot and Heitz Nitrous Acid-Chromic Acid Method.[62] Leucine or valine, in the absence of the other, is determined by transforming into hydroxy acid by *nitrous acid* and oxidation of the latter to acetone by *chromic acid.* By combination with another oxidation method and direct distillation, it is claimed that either leucine or valine may be determined in the presence of the other.

NOTE. Stakheeva-Kaverzneva[63] states that the method is not applicable to hydrolyzates containing both valine and leucine, even after the copper salts have been fractionated.

Van Slyke and Levene Lead Acetate Gravimetric Method.[64] The authors refer to the difficulties attending the separation of valine, leucine, and isoleucine owing to their inseparable isomorphous condition in mixtures and the nearly identical boiling point of their esters preventing fractionation. As obtained in Emil Fischer's laboratory, the three amino acids are grouped together. Ehrlich's method[65] is both tedious and inaccurate.

PROCESS. *Lead Acetate Precipitation.* In the present method, leucine is separated from valine by precipitation of the leucine in ammoniacal solution with *lead acetate solution* carefully regulated to avoid an excess. Make a preliminary determination of the carbon

content of the leucine-valine mixture, and calculate the amount of leucine present. Pulverize the mixture and mix in a flask with 7 parts of water. Heat the water to boiling, then remove from the flame and add 1.5 ml. of *ammonium hydroxide* for each gram of amino acid mixture. Stopper the flask and shake gently to dissolve the amino acids. From a graduated pipet run into the hot solution, dropwise *with continual rotation of the flask*, 4 ml. of 1.1 *M lead acetate solution* for each gram of leucine estimated to be present. The amount of lead acetate added is about 16% excess. Cool and let stand an hour. Filter the lead leucine salt, $Pb(C_6H_{12}O_2N)_2$, wash with 90% *ethanol*, then with ether.

Recovery of Valine. In the filtrate from the lead leucine precipitate the lead with *hydrogen sulfide*. Filter and evaporate the filtrate to dryness on a water bath. Take up the residue with a *3 + 1 ethanol-ether mixture* and wash with it in order to remove traces of acetic acid and ammonium acetate.

Recovery of Leucine. Dissolve the lead leucine in 15 to 20 parts of hot water plus one-fourth volume of *glacial acetic acid*. Remove the lead with *hydrogen sulfide*. Evaporate the filtrate to dryness and wash with *1 + 1 absolute ethanol-ether mixture* in order to remove traces of acetic acid.

Valine, Leucine, and Isoleucine

Block, Bolling, and Kondritzer Micro Method.[66] Apparatus. *Colorimeter*, with filter 520 μ.

Reagent. *Denigès Reagent*. Dissolve 5 g. of HgO in 20 ml. of H_2SO_4 and 80 ml. of water.

Process. *Hydrolysis*. Reflux with 10 volumes of *8 N sulfuric acid* and remove the acid by precipitation with *barium hydroxide*. Centrifuge, decant, and thoroughly wash the barium sulfate with hot water. Evaporate the filtrate and washings *in vacuo*, transfer to a 50-ml. volumetric flask, and make up to the mark.

Deamination. Remove 5- or 10-ml. aliquots with a pipet and deaminate with an excess of *sodium nitrite*, adding a few drops of *sulfuric acid* to maintain the acid reaction to *Congo paper*. Destroy the excess of nitrous acid by heating on the steam bath for 10 to 15 minutes.

Oxidation. Two different oxidizing agents are substituted for one under different conditions of the original method:

A. Dissolve 2 g. of *potassium dichromate* in 20 ml. of *5 N sodium acetate buffer* at *p*H 4.7 in a 3 x 20 cm. test tube, aspirate the acetone and ethylmethylketone formed into 2 water traps connected in series and cooled in an ice bath. Boil the oxidizing mixture and introduce the solution of the hydroxy acids from a buret at such a rate as to maintain a constant volume; this requires about 30 minutes. Continue the aeration and boiling until the volume is reduced to 10 ml.; this requires 30 minutes additional. Combine the aqueous solutions in the traps and dilute to volume.

Yield: Under these conditions of oxidization, valine yields 47 and leucine 6% of the theoretical amount of acetone and isoleucine 52% of the theoretical amount of ethylmethylketone.

B. Dissolve 2 g. of *potassium permanganate* in 20 ml. of the *M phosphate buffer* at *p*H 6.8 and conduct the oxidation as described under *A*.

Yield: Under these conditions, valine yields 50 and leucine 36% of the theoretical amount of acetone and isoleucine 52% of the theoretical amount of ethylmethylketone.

Leucine and Valine. Determine the acetone obtained in the dichromate and permanganate distillations by precipitation with *Denigès reagent* or colorimetrically by condensation with *10% ethanolic salicylaldehyde*.[67] Under these conditions, ethylmethylketone gives no color.

CALCULATION. Employ the following equations:

$$V = 4.85D - 0.81P$$

$$L = 7.54(P - D)$$

in which V, L, D, and P are the amounts respectively of valine, leucine, acetone by the dichromate oxidation, and acetone by the permanganate oxidation.

ISOLEUCINE. Two procedures should be employed:

A. Precipitate acetone with *Denigès reagent* from a portion of the aqueous solution from either the dichromate or permanganate oxidation of the hydroxy acids. Dilute the filtrate to a volume such that aliquots of 1 or 2 ml. will contain 0.005 to 0.05 mg. of ethylmethylketone. Dilute such aliquots in a colorimeter tube to 8 ml., add 4 ml. of *absolute ethanol*, cool to 0°, then add 4 ml. of *sulfuric acid*, and mix.

Color Formation. Add to the mixture 2 ml. of *10% ethanolic salicylaldehyde solution.* Stopper the tube, mix, and heat in a water bath at 50° overnight.

Color Reading. Take the reading, using a 520 μ filter.

CALCULATION. Prepare a calibration curve showing a range of 0.005 to 0.05 mg. of ethylmethylketone; 1 mg. of isoleucine = 3.5 mg. of ethylmethylketone.

B. Although acetone produces a color with salicylaldehyde in the presence of sulfuric acid, the intensity is much less than that formed with ethylmethylketone, hence the latter may be estimated directly in the presence of acetone from the calibration curves.

EXAMPLES. In a mixture of leucine, isoleucine, valine, and glycine in the proportions 24 : 6 : 8 : 250, the recovery was leucine plus 2%, valine plus 7%, and isoleucine plus 6% for bichromate oxidation and plus 1% for permanganate oxidation above the amounts expected from the formulas.

LEUCINE

Laine Ninhydrin Micro Method.[68] PROCESS. *Distillation.* Distil for 15 minutes 10 ml. of a solution containing 0.2 to 5 mg. of leucine, 7.5 g. of *ammonium sulfate*, 0.5 g. of *citric acid*, and 4 ml. of *1% ninhydrin solution*, catching the distillate in a U-tube containing 5 ml. of *1% sodium bisulfite solution.*

Titration. Pass steam through the distillate until the volume reaches 25 ml. and titrate with *standard iodine solution.*

ASPARTIC ACID

Arhimo Dinitrophenylhydrazine Distillation Method.[69] The method outlined below is an adaptation of the Pucher, Vickery, and Wakeman malic acid method[70] to the determination of aspartic acid. Malic acid must first be removed by ether extraction. Addition of nitrous acid is unnecessary since the *bromine water* added in the bromination is sufficient to oxidize aspartic acid to malic acid. Tyrosine and dihydroxyphenylalanine yield compounds volatile with steam which precipitate with *dinitrophenylhydrazine*, whereas glutamic acid does not. Malic acid is probably converted to dibromomalic acid which is oxidized by potassium permanganate to dibromooöxalacetic acid which is volatile with steam. (Although published in English, the journal is not available.)

ASPARTIC ACID AND GLUTAMIC ACID

Although glutamic acid in point of amount is the principal amino acid of many proteins, the dependable methods are chiefly of the isolation type such as the Fischer ester method and the Jones and Moeller method. The latter (U. S. Department of Agriculture) in the form here given is as applied to edestin.

Jones and Moeller Barium Hydroxide–Cupric Carbonate Method.[71] PROCESS. *Hydrolysis.* Heat 50 g. of the protein with 200 ml. of *20% hydrochloric acid* on a sand bath until nearly dissolved, then transfer to an oil bath and reflux for 30 to 36 hours. Filter from the suspended humin through asbestos and concentrate the dark-colored solution at below 50° to remove all possible hydrochloric acid.

Phosphotungstic Acid Precipitation. Remove the diamino acids, as directed by Van Slyke, and remove the phosphotungstic acid from the filtrate by shaking with *1 + 1 amyl alcohol-ether mixture,* then evaporate the nearly colorless solution of the monoamino acids to a thick sirup.

Barium Hydroxide Precipitation. Dissolve the sirup in 300 ml. of water, cool, and add *moist recrystallized barium hydroxide* until slightly alkaline to *litmus paper*. Remove by filtration the small amount of brown humin-like substance and wash several times with hot water. Add *barium hydroxide* to the filtrate until a small amount remains undissolved after shaking for several minutes. Pour the solution, which measures about 600 ml., with stirring into 5 volumes of *ethanol* and allow to stand 2 days. Filter and wash the barium salts with *ethanol*. Redissolve in 350 ml. of water and reprecipitate with 400 ml. of *ethanol*, then allow to stand in the refrigerator overnight. Filter with the aid of suction. The alcoholic filtrate consists chiefly of glycine.

The second precipitation removes nearly all the dicarboxylic acids, whereas most of the tyrosine and glycine and small amounts of bases that had precipitated with the dicarboxylic acids in the first precipitation remain in solution.

Glutamic Acid Separation. Decompose the main precipitate of barium salts with a slight excess of *sulfuric acid*, filter, and evaporate the filtrate and washings from the barium sulfate to about 800 ml. Remove the sulfuric acid with *barium chloride*, filter, and evaporate the filtrate to small volume, then saturate with *hydrochloric acid gas*. Separate the glutamic acid by filtration. The amount thus obtained is about seven-eighths of the total amount. Recover the remainder from the ethanolic filtrate from the barium salts and from the filtrate from the cupric aspartate.

Aspartic Acid Separation. Evaporate the filtrate and washings from the glutamic acid hydrochloride to a sirup to remove the greater amount of the hydrochloric acid. Remove the remainder of the chlorine by shaking with an excess of *silver sulfate solution*. Filter and evaporate the filtrate. Treat the residue with a small amount of *redistilled glacial acetic acid*. This dissolves hydroxyglutamic and pyrrolidonecarboxylic acids, which constitute nearly all the sirupy material in most protein hydrolyzates. The absence of these acids at this stage shows that hydroxyglutamic acid is not a constituent of the edestin molecule, thus confirming the conclusion of Osborne, Leavenworth, and Nolan.[72]

Cupric Aspartate Precipitation. Dissolve the remainder of the residue in water, heat to boiling, and precipitate the cupric aspartate with *cupric carbonate*. Filter, wash with cold water, dry at 125° under 25 mm. pressure, and weigh.

CALCULATION. The air-dry cupric aspartate contains 4.5 molecules of water which is driven off at 125°. The factor for converting dry cupric aspartate to aspartic acid is 0.672.

EXAMPLES. Glutamic acid and aspartic acid respectively: glycinin 18.5 and 9.4, edestin 19.2 and 10.2, ovalbumin 13.3 and 6.2, lactalbumin 12.9 and 9.3, stizolobin . . . and 9.2, fish muscle (halibut) 13.7 and 8.0, ox muscle 13.4 and 5.9, shrimp muscle 15.0 and 7.0, wheat gliadin 43.0 and 0.8, wheat glutenin 25.7 and 2.0, arachin 19.5 and 5.6, and kafirin 21.2 and 2.3%.

Glutamic Acid

Cohen Succinic Acid Manometric Method.[73] The method, devised at Sheffield University, permits determinations of as little as 0.1 mg. of glutamic acid. It depends upon changing the glutamic to succinic acid by treatment with an excess of chloramine T and subsequent hydrolysis [74] as follows:

$$(1)\quad \underset{\text{Glutamic acid}}{COOH\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH} + \underset{\text{Chloramine T}}{2C_6H_4(CH_3)SO_2\cdot NHCl} \rightarrow$$

$$\underset{\beta\text{-Cyanopropionic acid}}{COOH\cdot CH_2\cdot CH_2\cdot CN} \cdot CO_2 + 2HCl$$

$$\underset{p\text{-Toluenesulfonamide}}{C_6H_4(CH_3)SO_2\cdot NH_2}$$

$$(2)\quad \text{Cyanopropionic acid}$$

$$\underset{\text{Succinic acid}}{COOH\cdot CH_2\cdot CH_2\cdot COOH} + NH_4Cl$$

The succinic acid is then determined manometrically by the Krebs method,[75] following the principle of Szent-Györgi-Gözsy [76] that succinic acid extracted by ether may be determined by measuring the oxygen necessary for oxidation of succinate to fumarate in the presence of succinic dehydrogenase.

APPARATUS. *Kutscher-Steudel Continuous Extractor.*[77]

Manometer.

Respirometer, with conical cup, with side arm, large enough to hold 1 to 1.5 ml., and center chamber.

REAGENTS. *Citric-Citrate Buffer* (pH 4.7). Dissolve 8.40 g. of $C_6H_8O_7\cdot 1H_2O$ and 17.65 g. of $Na_3C_6H_5O_7\cdot 2H_2O$ in water and dilute to 50 ml.

Phosphate Buffer, 0.1 M (pH 7.4). Dissolve 17.8 g. of Na_2HPO_4 in about 500 ml. of water, add 20 ml. of 1.0 N HCl, and dilute to 1 liter.

Chloramine T Solution, 10%. Prepare immediately before use.

Succinoxidase. In order to avoid the increasing blank O_2 uptake on storage noted by Krebs,[78] Cohen proceeds as follows. Finely mince about 50 g. of pigeon muscle, wash with 500 ml. of water, filter by suction through muslin, and repeat twice. Suck as dry as possible after the third washing. For use, suspend a portion of the preparation in 4 to 5 times its weight of 0.1 M phosphate buffer (pH 7.4). For storage, moisten with distilled water and keep in the refrigerator in tightly glass-stoppered bottles. The preparation will remain active, without increasing its blank O_2 uptake, for 7 to 10 days.

PROCESS. *Deproteinization.* When tissue slices are used for analysis, deproteinization is not necessary since the small amount of protein present does not interfere. With suspensions of minced tissues, add two-thirds volume of *⅔ N sulfuric acid* and one-third volume of *10% sodium tungstate solution.*

Chloramine T Oxidation. Bring an aliquot to pH 4.7 by adding 1 to 1.5 ml. of *citric-citrate buffer.* Add 2 ml. of *10% chloramine T* and mix by shaking, then place in a rack and shake at 40° for 10 minutes. Precipitate most of the p-toluene sulfonamide formed and most of the unused chloramine T by placing in an ice bath for 15 to 20 minutes. Filter and wash the precipitate with several small volumes of water, then collect the filtrate and washings in a large test tube (25 x 200 mm.).

β-Cyanopropionic Acid Extraction. If glutathione is present, extract with ether the β-cyanopropionic acid leaving the glutathione derivative in the aqueous layer.

Acidify the above filtrate with 4 ml. of *10% sulfuric acid* and extract in a continuous extractor of the Kutscher-Steudel type, 1 to 2 hours usually being sufficient. Add 2.5 ml. of *phosphate buffer* to the extraction flask and slowly distil off the ether. Chill the remaining aqueous solution to precipitate the p-toluene sulfonamide, filter, and collect the

filtrate containing the β-cyanopropionic acid in test tubes.

β-Cyanopropionic Acid Hydrolysis. To the filtrate add *hydrochloric acid* to a final concentration of not less than 12.5%. Place the test tubes in boiling water for 15 minutes, remove, and cool. Add *sodium hydroxide solution* dropwise until the solution becomes hot; then add 0.5 ml. of *5% ammonium chloride solution* and mix well. (The ammonium chloride decomposes traces of chloramine T which, if present, decolorizes the indicator.) Add a few drops of *phenol red* and *alkali* to a purple color, avoiding an excess of alkali. Cool and transfer to the Kutscher-Steudel extractor.

Ether Extraction. Extract the cooled alkaline solution with *freshly distilled peroxide-free ether* for a time sufficient to remove traces of *p*-toluene sulfonamide (usually 1 to 2 hours), then remove the extraction flask, acidify strongly to *phenol red* (light yellow-pink color) with 3 ml. of *10% sulfuric acid*. Attach a clean extraction flask with fresh *ether* and continue the extraction 2 hours more. Phenol red is included in the extract.

Treatment of Succinic Acid Solution. To the ether solution, containing the succinic acid, add 2 to 3 ml. of *0.1 M phosphate buffer*, then dropwise *2 N sodium hydroxide solution* until the aqueous solution assumes the color of neutral or slightly alkaline phenol red. Distil off the ether, concentrate the alkaline aqueous solution on a steam bath to about 1 ml., transfer to a small graduated cylinder by means of a pipet, rinse the extraction flask with 0.5 to 2 ml. of *0.1 M phosphate buffer*, and combine the washings and solution.

Succinic Acid Determination. Place 4 ml. of the *succinoxidase* in the main part of the conical cup of the manometer, an aliquot of the succinic acid solution (0.5 to 1.5 ml.) in the side arm, and 0.2 ml. of *2 N sodium hydroxide solution* in the center chamber. Make a blank to correct for the oxygen uptake of the enzyme which should be only about 1 or 2 $\mu l \cdot O_2$ per milliliter of enzyme per hour. Further details are given in Krebs' paper.

CALCULATION. The $\mu l \cdot O_2$ uptake observed is converted into milligrams of glutamic acid (G) as follows:

$$112 \quad \times 1.47$$

EXAMPLES. Pigeon breast muscle 34, pigeon gizzard 94, sheep brain 80, and sheep heart 171 mg. of glutamic acid per 100 g., wet basis.

Arhimo and Laine Silver Succinate Volumetric Method.[79] PROCESS. A. GLUTAMIC ACID ONLY. *Deaminization.* To 5 ml. of the solution containing 1 to 10 mg. of glutamic acid, add 1 ml. of *2 N sulfuric acid* or *nitric acid* and 2 ml. of *30% sodium nitrite solution*, shake, and allow to stand 10 minutes at room temperature.

Oxidation to Succinic Acid. Boil to remove the excess of nitrous acid, cool, and add an excess of *1.5 N potassium permanganate solution.* After letting stand 1 hour, filter and wash.

Extraction. Transfer the filtrate to a continuous extractor and extract with *ether* for 48 hours.

Silver Succinate Precipitation. Evaporate the ether, take up in *ethanol*, neutralize with *sodium hydroxide solution*, and precipitate the succinic acid with *saturated silver nitrate solution in ethanol.* Filter and wash.

Titration. Dissolve the precipitate in *dilute nitric acid* and titrate with *standard 0.005 N ammonium thiocyanate solution.*

B. OTHER AMINO ACIDS. Since certain monocarboxylic acids also form succinic acid on oxidation, precipitate the glutamic acid by the Foreman method.[80]

Dissolve the precipitate containing 25 to 500 mg. of glutamic acid in 50 ml. of *N sulfuric acid* or *nitric acid*, add 5 ml. of *30%*

sodium nitrite solution, oxidize, extract, precipitate the silver salt, and filter as above. The silver salt may be weighed or titrated with *standard 0.1 N ammonium thiocyanate solution*.

CALCULATION. Use: 1 ml. of 0.005 N and 0.1 N ammonium thiocyanate solution = respectively 0.368 and 7.36 mg. of glutamic acid.

ARGININE

Methods Based on the Decomposition of Arginine by Enzymes. Arginase, extracted from the livers of dogs or cats, decomposes arginine into carbamide (urea) and ornithine thus:

$$\underset{\text{Arginine}}{NH{:}C(NH_2)NH\cdot(CH_2)_3\cdot CH(NH_2)COOH}$$

$$\underset{\text{Urea}}{CO(NH_2)_2} + \underset{\text{Ornithine}}{NH_2\cdot(CH_2)_3\cdot CH(NH_2)COOH}$$

If the solution is then digested with urease, prepared from the soy bean, or preferably the jack bean, ammonium carbonate is obtained.

These reactions form the basis of a number of methods which, being simple and accurate, would doubtless be more in general use were it not for the difficulties of obtaining and keeping the enzymes.

Jansen Arginase-Urease Distillation Method.[81] REAGENTS. *Arginase Extract.* Soak dog or cat liver in water to remove blood, then add toluene and grind with quartz sand. Separate the liquid from the solid matter by centrifuging, dialyzing in a stream of water, and precipitate 3 parts of the solution of arginase with 2 parts of ethanol and 1 part of ether. Remove as much of the water as possible by centrifuging and suspend in water, together with toluene, and keep in the refrigerator. The suspension keeps 3 months.

Urease Extract. Prepare a water extract of the enzyme from ground soy bean and mix with an equal volume of glycerol.[82]

PROCESS. *Hydrolysis.* Reflux 1 g. of the protein with 10 ml. of *hydrochloric acid* for at least 24 hours (overnight is not sufficient) or until all the arginine is hydrolyzed, and evaporate the hydrolyzate until nearly all the acid is removed. Take up the residue in water and add *sodium hydroxide solution* until the solution is faintly alkaline to *litmus* or *neutral red*. Transfer to a 50-ml. volumetric flask, make up to the mark, and mix.

Enzyme Digestion. Pipet three 15-ml. portions of the hydrolyzate into large test tubes. Use one portion for the digestion with the *urease*, the other two for the digestion with *urease* plus *arginase*. Continue both digestions for 24 hours, after which liberate the ammonia by addition of *potassium carbonate*, distil under reduced pressure into *standard acid*, and titrate the distillate with *standard alkali*.

The ammonia obtained in the urease procedure is derived from any urea that may be present in the sample, as well as from the protein during hydrolysis, whereas the ammonia obtained in the urease-arginase procedure includes also that derived from arginine. The difference represents ammonia derived from arginine only.

I. Bonot and Cahn Modification.[83] In this modification, *arginase* is used in conjunction with *xanthydrol* with accurate results.

II. Hunter and Dauphinee Modification.[84] After studies at the University of Toronto, the following details were adopted.

APPARATUS. *Incubator.*

REAGENTS. *Standard Crude Arginase Extract.* Mix a weighed quantity of finely ground fresh liver with as many milliliters of 75% glycerol as there are grams of the tissue. Shake well for 10 minutes and heat in a water bath at 62 to 65°, shaking frequently, for 5 minutes after the contents reach 58°. Cool under the tap and filter on a large pleated Chardin paper. From 1500 g. of liver, 1 liter of clear red filtrate should be obtained in 12 hours. Adjust to pH 7 by

adding NaOH and store in a refrigerator. The activity should range from 80 to 100 units per milliliter.

Urease Extract. Glycerol extract of Jack bean.

A. Direct Determination on Protein Hydrolyzate Without Removal of Humin and Ammonia (for lightly pigmented hydrozylate). *Hydrolysis.* Boil 10 g. of the protein for 5 to 24 hours with 10 to 20 times its weight of *20% hydrochloric acid.* Evaporate *in vacuo* to a thick sirup to remove as much acid as possible, dissolve the residue in water, nearly neutralize with *sodium hydroxide solution,* and make up to a solution of which 5 or not more than 10 ml. contain 6 to 12 mg. of arginine nitrogen, the concentration in terms of the original protein being 3 to 15%. Filter the hydrolyzate and remove a suitable aliquot for total nitrogen determination.

(*a*) *Deaminase Absent.* Pipet 5-ml. aliquots into four Van Slyke-Cullen urea tubes. Reserve two for the determination of ammonia nitrogen (amide blank).

To the other pair (principal tubes) add successively 2 ml. of *0.25 M disodium phosphate solution,* a drop of *phenolphthalein indicator,* and, from a buret, *1.0 N sodium hydroxide solution* sufficient to produce a light pink color, then 1 ml. of the *active arginase extract* and a few drops of *toluene.* Cover with tinfoil, place in water bath at 37° for 12 to 24 hours. At the end of the digestion, add to each tube 1 to 2 drops of *phenol red solution* and dropwise sufficient *1.0 N hydrochloric acid* just to discharge the pink color.

Meanwhile in each of a third pair of tubes (enzyme blanks) place 3 ml. of water and 1 ml. of *arginase extract.*

Set up all six tubes for aeration, add 1 ml. of *urease solution* to the principal tubes and to the enzyme blanks. After the interval allowed for urease action, complete the determination. In the principal tubes, use at least 9 ml. of *saturated potassium carbonate solution,* for the liberation of ammonia, and 10 ml. of *N/14 hydrochloric acid* (or 50 ml. of *N/70*) for its reception, but with the other tubes reduce each of these quantities to 5 ml.

(*b*) *Deaminase Present.* If a deaminizing arginase is to be used, substitute for the simple amide blank a deaminase blank and add an arginase blank for determination of preexistent ammonia.

Treat four 5-ml. portions each of a nearly neutral hydrolyzate, prepared as above, in urea tubes with 2 ml. of *0.25 M disodium-monohydrogen phosphate solution,* add *phenolphthalein,* and titrate with *1.0 N sodium hydroxide solution* to a light pink color. Add to each of the four tubes 1 ml. of *arginase extract* and a little *toluene,* then heat at 37° for 12 to 24 hours. After the incubation, add to each tube a drop or two of *phenol red solution* and titrate with *1.0 N hydrochloric acid* to a *p*H of about 6.8.

Add to two of the four (principal tubes) 1 ml. of *urease extract* but not to the other two (deaminase blanks). After incubating the proper time for the urease action, determine ammonia in the four tubes as above.

At the same time or earlier, perform an enzyme blank; also an arginase blank in which the ammonia is determined on 5 ml. of the liver extract by the Thomas and Van Hauwaert Potassium Carbonate-Aeration Method (Part II, H5).

B. Determination in a Solution of Bases Precipitated by Phosphotungstic Acid. There are several drawbacks to this procedure, although these have, in a measure, been overcome. Details are given in Hunter and Dauphinee's paper.

III. Ranganathan Conductivity Modification.[85] The procedure is briefly as follows: 1 ml. of the arginine solution together with 0.5 to 2.0 ml. of *0.06 M phosphate buffer* at *p*H 7 is made up to 4 ml. in a conductivity cell and kept at 30° for 30 minutes. When the resistance becomes steady, add to the

solution 2 ml. of a dialyzed mixture of *arginase* and *urease* and measure the conductivity after 30 minutes.

The estimation requires only 50 mg. of the protein and less than 1 hour. The results are stated to be accurate within 1% for 0.5×10^{-4} g.

Methods Based on Precipitation with Flavianic Acid. Flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid), $C_{10}H_4(NO_2)_2 \cdot SO_3H \cdot 3H_2O$, forms with arginine pale yellow diflavianate or golden yellow monoflavianate. Following Kossel and Gross, the discoverers, Kossel and Staudt [86] showed that the method is quantitative at acidities between the turning point of litmus and 0.1 *N* sulfuric acid. An equal amount of histidine does not interfere. The content of arginine nitrogen determined as flavianate subtracted from that of arginine plus histidine in the silver-baryta precipitate obtained by the Kossel and Kutscher method gives the histidine content.

Kossel and Gross Flavianic Acid Gravimetric Method.[87] PROCESS. *Hydrolysis.* Boil 250 g. of the protein for 18 hours with *33% sulfuric acid,* then remove most of the free acid with *barium hydroxide solution.*

Flavianic Acid Precipitation. Add *concentrated flavianic acid solution* (4 g. for each gram of arginine) and allow to stand 2 days, with stirring, especially during the first few hours, to avoid formation of a hard crystalline mass; then filter, and wash. Transfer the precipitate to a flask and digest on a water bath for 1 to 2 hours with 100 to 150 ml. of a *very dilute aqueous flavianic acid solution.* After 3 to 8 days, filter on a Gooch crucible, wash with cold water, dry at 95 to 100°, and weigh.

Kossel and Staudt Silver-Barium Flavianic Acid Method.[88] The method is a combination of the Kossel and Kutscher method, so far as the separation of histidine and arginine from other bases is concerned, and the Kossel and Gross flavianic acid method for the determination of arginine. Due regard is given the solubility of arginine in concentrated barium hydroxide solution (36 mg. per liter) and the practical insolubility of histidine, also the high histidine content of certain protamines, especially sturin. Silver nitrate is substituted for silver sulfate, except in special cases. If the sulfate is used, a second precipitation is necessary because its lesser solubility increases the bulk of liquid.

PROCESS. *Solution.* Prepare an aqueous solution of the hydrolyzate containing in 50 ml. arginase equivalent to about 0.5 g. of the carbonate.

Precipitation with Flavianic Acid. To a 10-ml. aliquot, add 0.5 g. of *flavianic acid dissolved in 10 ml. of water* and dilute to 30 ml. Allow to stand 3 days, collect the precipitate on a Gooch crucible, and wash with water containing a trace of flavianic acid until the yellow color of 5 to 10 ml. of the wash solution remains constant. Dry the precipitate at 105° and weigh.

Vickery Modification.[89] PROCESS. *Hydrolysis.* Hydrolyze 3 to 5 g. of the protein with *20% hydrochloric acid* and remove as much as possible of the acid by repeated evaporation *in vacuo.* Decolorize with *norite.* Filter and make up the filtrate to 250 ml.

Diflavianate Precipitation. To 50 ml. of the hydrolyzate add at room temperature 4 to 5 moles of *flavianic acid* for each mole of arginine present, as calculated from the best available information on the yield of arginine from the protein. (Vickery states that 1 g. of arginine requires 1.805 g. of flavianic acid for 1 mole.) Keep in the refrigerator for 4 days, stirring at least once daily.

The precipitate consists chiefly of pale yellow needles of the diflavianate, sometimes mixed with a little monoflavianate in the form of orange-yellow nodules formed by masses of plates, and also smaller amounts of ammonium flavianate and probably other flavianates.

Filter on a sintered glass funnel or crucible,

wash in 2 or 3 portions with about 30 ml. of *water saturated with arginine monoflavianate.* Although much of the diflavianate is changed to the monoflavianate and the filtrate is highly colored, there is no appreciable loss of arginine. The ammonium flavianate and other flavianates are largely removed.

Monoflavianate Precipitation. Attach the funnel or crucible containing the diflavianate to a 150-ml. suction flask and stir the precipitate with a little hot water. Add cautiously *5 N ammonium hydroxide* dropwise from a 1-ml. pipet graduated in tenths, avoiding more than enough to impart a faint odor. Stir until all the precipitate has dissolved, suck gently through sintered glass, then wash with hot water until color is no longer evident, using if necessary a trace of *ammonium hydroxide.* Transfer the filtrate and washing from the flask into the original beaker and heat to dissolve any crystals that remain in the beaker. Heat cautiously to boiling the solution, which should not exceed 40 ml., and add *1.0 N sulfuric acid* equivalent to or slightly in excess of the amount of *5 N ammonium hydroxide* used to dissolve the diflavianate.

The monoflavianate usually crystallizes at once in golden yellow lustrous plates which should not be disturbed by stirring until their formation is complete at room temperature. Keep in the refrigerator overnight and collect the precipitate the next day on a tared sintered glass crucible as before, washing several times with *water saturated with arginine monoflavianate* at room temperature, then with a little *ethanol.* Dry a few hours at 105°, cool in a desiccator, and weigh quickly.

CALCULATION. Multiply the weight of the arginine monoflavianate by 0.3566 to obtain the weight of arginine and by 0.1148 to obtain the weight of arginine nitrogen.

EXAMPLES. Results in percentages by Vickery by the modified flavianic acid, silver precipitation, arginase, and Van Slyke methods, are respectively as follows: edestin 14.50, 15.81, 15.50, and 15.71; casein 4.40, 3.85, 3.85, and 3.70; gelatin 9.34, 7.62, 8.90, and 8.34; gliadin (wheat) . . ., 2.92, 2.64, and 3.13; arachin 17.54, 12.54, . . ., and . . . By the silver precipitation method only, amandin 11.85, zein 1.60.

Methods Based on the Sakaguchi Reaction.[90] When *alkali* is added to a 3-ml. solution of a protein, followed by 2 drops of *0.1% α-naphthol in 70% ethanol* and a few drops of *5% sodium hypochlorite solution,* a deep red coloration is produced. This discovery of Sakaguchi has been made the basis of quantitative procedures for the determination of arginine by chemists in several countries. Weber substituted *sodium hypobromite* for the hypochlorite and compared the unknown with a series of standards prepared from solutions of arginine monohydrochloride as in the actual analysis. Jorpes and Thorén adopted the Weber method, except that they determined the color value photometrically as did Dumazert and Poggi, who substituted 0.25 ml. of *40% sodium carbonate solution* for 1 ml. of 10% sodium hydroxide solution.

Only arginine of the protein cleavage products gives the reaction, the color being due, it is stated, to the guanidine portion of the molecule.

Weber Naphthol Hypobromite Colorimetric Method.[91] The method was devised at the University of Kansas.

APPARATUS. *Colorimeter* or *Nessler Tubes.*

REAGENTS. *α-Naphthol Solution,* 0.02%. Dilute 20 ml. of 0.1% α-naphthol solution in ethanol to 100 ml. with water. Keep in an amber bottle.

Sodium Hypobromite Solution. Dissolve 2 g. of bromine in 100 ml. of 5% NaOH solution. Keep in an amber bottle.

Urea Solution, 40%.

Standard Arginine Monohydrochloride Solution. Prepare a stock solution of 0.1209 g. of the salt and 2 g. of sodium benzoate in 1

liter of 0.1 N HCl. Dilute 3 ml. to 100 ml. for use; 5 ml. = 0.015 mg. of arginine.

PROCESS. *Color Formation.* Place 5 ml. of the solution of the unknown and 5 ml. of the *standard arginine monohydrochloride solution* each in a 150 x 18 mm. test tube and cool in an ice bath at 5° 2 to 3 minutes. Without removal from the bath add 1 ml. of *10% sodium hydroxide solution* and 1 ml. of *0.02% α-naphthol solution.* Hold in the ice bath for 2 or 3 minutes, then without removal from the bath add 0.2 and 0.15 ml. respectively of *sodium hypobromite solution* to the unknown and the standard. Mix and within 5 to 10 seconds add 1 ml. of *40% urea solution.*

Color Comparison. Compare the unknown with the standard in Nessler tubes or a colorimeter.

I. Jorpes and Thorén Modification.[92] This procedure is identical with that of the Weber method, except that instead of a color comparison in Nessler tubes or a colorimeter, a reading is made in a photometer, using color filter S50. The weight of arginine is obtained from a curve plotted with milligrams of arginine as abscissas and readings of the photometer scale as logarithmic ordinates.

II. Dumazert and Poggi Modification.[93] This more recent modification of the Weber method is really a modification of the Jorpes and Thorén modification, since it differs materially from the latter only in that 0.25 ml. of *40% sodium carbonate solution* is substituted for 10 ml. of 10% sodium hydroxide solution. The reading is made in a Pulfrich photometer, using filter No. 7. The authors state that guanidine and its bisubstituted derivatives (creatine) give no coloration under the conditions of the method.

LYSINE

Ayre Silver-Picric Acid Gravimetric Method.[94] Although this method, developed at the University of Reading, introduces no novel reaction, it employs approved procedures for the separation of cystine and the members of the hexone group.

PROCESS. *Hydrolysis.* To 4 to 5 g. of the protein add five times its weight of *20% hydrochloric acid* and reflux for 36 to 40 hours. Transfer the hydrolyzate to a 250-ml. Pyrex centrifuge bottle, using a small amount of water for rinsing.

Phosphotungstic Acid Precipitation. Add to the hydrolyzate an excess of *saturated phospho-24-tungstic acid solution,* dilute to a final hydrochloric acid concentration of 5% by weight and allow to stand overnight at room temperature. On the next morning centrifuge, decant on a filter, and wash twice by stirring with 25-ml. portions of *2.5% phosphotungstic acid solution in 5% by weight sulfuric acid* and centrifuging. Sometimes a better separation is secured by stirring before the first centrifuging.

Phosphotungstic Acid Precipitate Decomposition. Disintegrate the phosphotungstic precipitate with 50 ml. of water to which has been added 1 ml. of *sulfuric acid* and a liberal amount of *amyl alcohol-ether mixture* as recommended by Van Slyke;[95] then centrifuge. Pour the clear liquids (both aqueous and solvent) into a separatory funnel and wash the residue consisting of acid-insoluble humin (the humin precipitated by the phosphotungstic acid, and ammonium phosphotungstate) twice as before, but use smaller amounts of *acidified water* and *amyl alcohol-ether mixture,* then add the washings to the mixed liquid in the separatory funnel. After a few minutes, draw off the aqueous solution (which should be water clear) and shake out three times successively with *amyl alcohol-ether mixture.* Shake the combined amyl alcohol-ether solution once with a small quantity of *water acidified with sulfuric acid* and wash the latter solution once with a fresh portion of *amyl alcohol-ether mixture.* Give the combined aqueous solution a final washing with a small quantity of *amyl alcohol-ether mixture.*

Instead of the foregoing procedure, decompose the phosphotungstic acid precipitate of blood meal with 50 ml. of *acidified water* and 75 ml. *amyl alcohol-ether mixture.* After centrifuging, siphon the aqueous layer from the solvent layer, wash the amyl alcohol-ether and the precipitate by stirring with three successive portions of 25 ml. of *acidified water*, centrifuging and siphoning the aqueous washings as before.

Run the final aqueous solution into a 500-ml. flask, evaporate *in vacuo* to small volume, and transfer to a 250 Pyrex centrifuge bottle. Heat in a boiling water bath and add *solid silver oxide* in small portions, with rapid stirring until an excess is present as shown by the brown precipitate with cold *saturated barium hydroxide solution*, the final volume for 5 g. of protein being less than 200 ml.[96] Cool the solution, add warm *saturated barium hydroxide solution* until distinctly alkaline to *Nile blue or alizarin yellow S*. Centrifuge and wash twice with small amounts of cold *saturated barium hydroxide solution.* Transfer the centrifugate and washings to a Büchner flask and acidify to *Congo red.*

Removal of Silver. Precipitate the silver with *hydrogen sulfide* under pressure. Centrifuge and thoroughly wash the precipitates of barium sulfate and silver sulfide with water. Concentrate the clear aqueous solution *in vacuo*, dilute to 100 ml., and remove 1-ml. aliquots for determination of total nitrogen.

Transfer the remainder of the solution to a 250-ml. centrifuge bottle, precipitate the bulk of the sulfuric acid with *barium hydroxide solution*, filter, and wash the barium sulfate with water. Evaporate the filtrate and washings to small volume *in vacuo* in the presence of a small excess of *barium carbonate.* Remove the barium carbonate and barium sulfate by filtration through a No. 42 Whatman filter and concentrate the filtrate *in vacuo* to 2 ml. Wash the detachable head of the bottle and the capillary with 5 to 10 ml. of *93% ethanol*, adding 1 or 2 drops of water if the mixture becomes more than faintly turbid.

Picric Acid Precipitation. For each milligram of nitrogen present, add half the quantity of *picric acid* required to form the lysine picrate. Stir the solution and keep in the refrigerator overnight; during this time the lysine picrate crystallizes. Collect the precipitated crystals on a sintered-glass crucible under reduced pressure, wash with cold *absolute ethanol*, and test the filtrate for lysine that escaped precipitation by adding *picric acid* dissolved in warm *absolute ethanol.*[97] Collect the additional crop of crystals (if any) on the sintered-glass crucible used before, dry the combined crop, and weigh.

Dissolve the crystals in hot water added to the crucible, wash with a little hot water, and concentrate the filtrate and washing until crystals form on the surface. After cooling in the refrigerator, collect the crystals on the sintered-glass crucible, wash with *absolute ethanol*, dry in a boiling water oven, and weigh as before. Concentrate the filtrate and washings to small volume, cool, and filter as before. Measure the final volume of mother liquid from the last crystallization and apply a correction of 3.4 mg. per ml. for the solubility of lysine picrate. The first crop should explode at 266 to 267°, the second crop above 250.[98]

Hydroxylysine

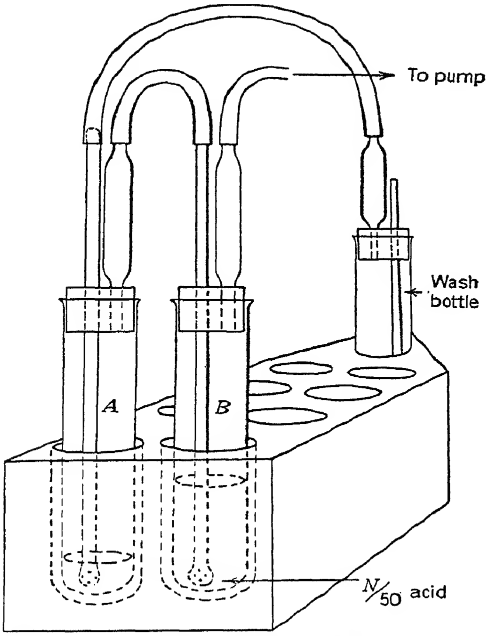
Van Slyke, Hiller, and MacFadyen Periodate Ammonia Method.[99] In the present procedure diamino acids are precipitated by phosphotungstic acid from protein hydrolyzates and hydroxylysine is determined in the precipitate by means of the ammonia liberated in accordance with the following general reaction involving sodium periodate:

$$RCH(OH)\cdot CH(NH_2)R' + NaIO_4 = RCHO + R'CHO + NH_3 + NaIO_3$$

Apparatus. *Van Slyke Amino Nitrogen Assembly.* See Van Slyke Nitrous Acid Gasometric Method above, Fig. 47.

Van Slyke and Cullen Ammonia Aeration Assembly (Fig. 52).

Carbon Dioxide-Free Air Reservoir. See Van Slyke, Dillon, MacFadyen, and Hamilton Ninhydrin Gasometric Method, above under Amino Acids.



Courtesy of the Authors and J. Biol. Chem. 1914, **19**, *217*

Fig. 52. Van Slyke and Cullen Ammonia Aeration Assembly.

Van Slyke and Neill Manometric Assembly (Fig. 53). The pipet is calibrated at two points to hold a ml. of gas and A ml. of total volume. The manometric method differs from the volumetric method previously employed in that the volume is brought to an arbitrarily chosen amount and the weight is determined from the pressure exerted on the manometer, whereas in the volumetric method the pressure is that of the atmosphere and the volume of gas is measured.

Reagents. *Phosphotungstic Acid.* Purify by Winterstein's method [100] as follows. Dissolve $20WO_3 \cdot 2H_3PO_4 \cdot 25H_2O$ in an equal amount of water and shake in a separatory funnel with enough ether to form 3 layers, the lowest being the ether solution of the acid, the middle aqueous, and the top the excess of ether. Remove the lowest layer, wash with 3 equal portions of water, and dry on the steam bath. Recovery about 70%.

Periodic Acid, 0.2 *M*. Dissolve 46 g. of $HIO_4 \cdot 2H_2O$ in water, dilute to exactly 1 liter, let stand overnight, filter, and standardize against 0.1 *N* arsenite solution.[101] The theoretical amount is 45.59 g. per liter, but using 46 g. makes allowance for hydroscopic moisture. A slight precipitate may settle on standing.

Bromine Solution (Van Slyke and Kugel). Dissolve 2.5 ml. of bromine in a solution of 60 g. of KBr in 100 ml. of water. Keep in a dark bottle and check at least monthly by titrating with thiosulfate solution.

Process. *Hydrolysis.* Reflux 3 g. of the sample—0.5 g. is sufficient for a single analysis without testing the diamino acid solution for ammonia—for 24 hours with about 30 times its weight of *6 N hydrochloric acid.* Expel the free hydrochloric acid *in vacuo,* then remove the ammonia by distillation with *calcium hydroxide in vacuo* and the melanin by absorption with *calcium hydroxide* by the Van Slyke Phosphotungstic Acid Comprehensive Method above. (If the periodate treatment (see below) is applied to one-tenth of the melanin filtrate, it will yield as ammonia the nitrogen of the threonine, serine, and hydroxylysine together.)

Diamino Acids Precipitation. Precipitate the diamino acids with *phosphotungstic acid* as in the Van Slyke Nitrous Acid Gasometric Method (which see), except that the precipitation is made (for 3 g.) in a larger volume

(300 ml. at 23 to 25°) and in a lower acid concentration (0.25 *N* hydrochloric acid). Filter after 48 hours and wash with suction 5 times, using 8-ml. portions of *5% phosphotungstic acid in 0.25 N hydrochloric acid.* To insure complete separation from the monoamino acids, recrystallize the diamino phosphotungstates as follows. Rinse the washed

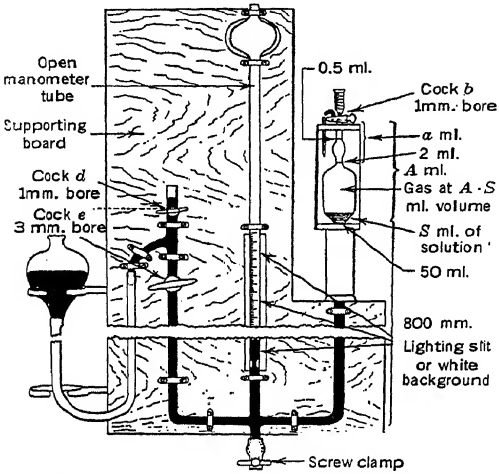


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FIG. 53. Van Slyke and Neill Manometric Assembly.

precipitate with water back into the precipitation flask and redissolve by adding *2 N sodium hydroxide solution* until neutrality to *alizarin red* is reached. Make up again to 300 ml., add 6 ml. of *hydrochloric acid* to about 0.25 *N*, heat, and add 15 g. of *phosphotungstic acid solution.* After 48 hours, again filter, and wash as directed in the Van Slyke Method.

Rinse the precipitated diamino acids into a 30-ml. volumetric flask, add a drop of *alizarin red* and *5 N sodium hydroxide solution* with stirring until the precipitate dissolves and the solution is neutral to the indicator, and finally make up to 30 ml.

Periodate Treatment. Into the receiving tube of the Van Slyke-Cullen ammonia assembly (Fig. 52) place 12 ml. of approximately *0.1 N sulfuric acid* and into the reaction tube place in the order given 5 ml. of the diamino acid solution (unknown) containing preferably hydroxyamino acid to yield 0.1 to 2 mg. of ammonia nitrogen, 1 ml. of *5% glycine solution,* 1 drop of *caprylic alcohol,* 1 ml. of *2 N sodium hydroxide solution,* 2 ml. of *0.2 M periodic acid,* and quickly 10 ml. of *saturated potassium carbonate solution.*

Aeration. Immediately after adding the carbonate close with the stopper bearing the inlet and outlet tubes for the air current. Run the air current at first 2 minutes at about 1 bubble per second, then accelerate gradually until after 5 minutes the rate is 4 liters per minute, continuing at this rate for 25 minutes. Several tubes may be aerated together.

Bromine Treatment. Using a rubber-tipped pipet, transfer 10 ml. of the solution in the receiving tubes to the chamber of the Van Slyke-Neill manometric assembly (Fig. 53). To remove the air from the solution, lower the mercury to the bottom of the chamber, shake the latter for 2 minutes, and eject the air, repeating the operation to remove the last traces. Mix in the cup of the chamber 1.25 ml. of the *10 N sodium hydroxide solution* and 0.75 ml. of the *bromine solution* and draw 1.5 ml. of the mixture down into the chamber.[102]

Reading. Lower the mercury in the chamber to the 50-ml. mark, shake the chamber for 3 minutes to extract the nitrogen formed by the action of the hypobromite on the ammonia. Bring the gas volume to 2 or 0.5 ml., according to the amount, and take the reading (P_1) on the manometer, then eject the gas, return the water meniscus to the 0.5- or 2-ml. mark, and repeat the reading (P_0) on the manometer.

Blank. Carry through the operation, including aeration, as in the actual analysis, except that the 5 ml. of the unknown are re-

placed by 5 ml. of ammonia-free water, thus obtaining P_1 and P_0.

CALCULATION. Obtain the milligrams of ammonia nitrogen (A) by the formula

$$A = 1.2(P_1 - P_0 - C) \times F_1 \times F_2$$

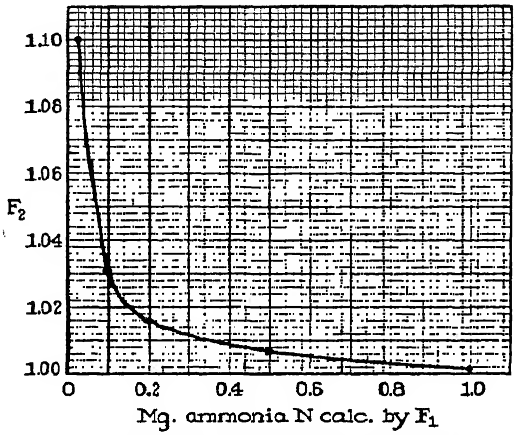
in which 1.2 corrects for only 10 of the 12 ml. of 0.1 N acid being used, C is $p_1 - p_0$, and F_1 is the factor for calculating ammonia nitrogen from the pressure of 2 ml. of the nitrogen yielded by the hypobromite reaction obtained from the table below.[103] If the measurement is made with the gas at 0.5 ml., divide the 2-ml. factor by 4. The factor F_2 (used only when the ammonia nitrogen is less than 0.5 mg.) is a correction, obtained from the graph in Fig. 54, necessitated by the yield of nitrogen per milligram of ammonia being lower than indicated by F_1.

VAN SLYKE FACTORS FOR CALCULATION OF AMMONIA NITROGEN FROM THE PRESSURE OF 2 ML. OF NITROGEN

(The empirical correction factor 1.04 for the reaction with hypobromite is included.)

Temp. °C.	F_2	Temp. °C.	F_2
15	0.003250	25	0.003136
16	241	26	124
17	227	27	116
18	215	28	004
19	201	29	092
20	192	30	080
21	180	31	071
22	168	32	060
23	156	33	050
24	145	34	040

Alternative Measurement of the Ammonia by Titration. The procedure of aeration is the same, except that instead of 12 ml. of 0.1 N sulfuric acid the receiving tube is charged with *exactly 15 ml. of 0.01 N sulfuric acid* and 1 drop of *1% alizarin red solution*. After the aeration, the excess of 0.01 N acid is titrated back with *0.01 N sodium hydroxide solution* freshly prepared by dilution of 0.1 N. The calculation is: mg. of ammonia $N = 0.14(15 - B - C)$, where B = ml. of 0.01 N NaOH used and $C = 15 - B$ determined in a blank analysis.



Courtesy of the Authors and J. Biol. Chem. 1941, **141,** *689*

FIG. 54. Van Slyke, Hiller, and MacFadyen Graph.

CYSTINE AND CYSTEINE

Folin and Looney Phosphotungstic Colorimetric Method.[104] This method as formulated at the Harvard Medical School has had wide application.

APPARATUS. *Colorimeter.*

REAGENTS. *Folin and Denis Uric Acid Reagent.*[105] Reflux for 2 hours 100 g. of sodium tungstate and 80 ml. of 85% H_3PO_4 in 750 ml. of water. Cool and make up to 1 liter.

Standard Cystine Solution, containing 5% H_2SO_4 and 1 mg. of cystine per milliliter. The solution keeps indefinitely.

PROCESS. *Hydrolysis.* Reflux in a 300-ml. Kjeldahl flask, fitted with a Hopkins

condenser, 1 to 5 g. of the dry protein with 25 ml. of *20% sulfuric acid* for 12 hours over a micro burner, cool, dilute to 100 ml. in a volumetric flask, and thoroughly mix.

Color Formation. Pipet 1 to 10 ml. of the hydrolyzate into a 100-ml. volumetric flask, add 20 ml. of *saturated sodium carbonate solution* and 10 ml. of *20% sodium sulfite solution*, shake well, and let stand while preparing the standard solutions.

To prepare the standards, add to 1 and 3 ml. respectively of *cystine solution* or 1 and 3 mg. of *cystine*, the same reagents in the same amounts as to the unknown, shake, and let stand for 5 minutes.

To each standard and the unknown, add, with shaking, 3 ml. of the *Folin and Denis uric acid reagent*, let stand 10 minutes, fill to the mark, and mix.

Color Comparison. Match the color of the solutions. There is no need for undue haste as the fading is the same in both standard and unknown.

I. Hunter and Eagles Lithium Sulfate Modification.[106] The description of the method given by its originators (Toronto University) is remarkable for its brevity and clarity.

Reagent. *Phosphotungstic Acid Reagent.*[107] Hunter and Eagles omit the Li_2CO_3 employed in the Folin and Trimble reagent.[85] (A) To 20 ml. of 85% H_3PO_4, add 80 ml. of water, heat to boiling, add 80 g. of ordinary $20WO_3 \cdot 2H_3PO_4 \cdot 25H_2O$, boil gently for 1 hour, and cool. (B) Dilute 65 ml. of 85% H_3PO_4 with 200 ml. of water. Mix A and B and dilute to 1 liter.

Process. A novel feature is the tabulation showing the volume and strength of the reagents, the intervals, and the readings of unknown and standard. The order is (1) milliliters of cystine as tabulated below; (2) milliliters of *N sodium hydroxide solution* (same as of cystine); (3) milliliters of water required to bring (1) plus (2) to 1 ml.; (4) 0.5 ml. of *5% lithium sulfate solution;* (5) 2 ml. of *1.0 N sodium hydroxide solution* (free from CO_2); (6) 2 ml. of *5% sodium sulfite solution;* (7) interval of 1 minute; (8) 0.5 ml. of *uric acid reagent;* (9) interval of 5 minutes; (10) 14 ml. of water to bring the volume to 20 ml.; (11) with unknown set at 15.0 mm., reading of standard in millimeters equivalent to milliliters of cystine solution as tabulated below.

The table which follows shows the number of milliliters of cystine solution (1 ml. = 1 mg.) in 20 ml. corresponding to the reading of the standard solution of 0.2 mg. of cystine in 20 ml. with test solution set at 15 mm.

Cystine Solution ml.	Standard Reading mm.
0.05	3.6
0.10	7.5
0.15	11.2
0.20	15.0
0.25	18.7
0.30	22.4
0.35	26.2
0.40	30.1

II. Folin and Marenzi Modification.[108] As cysteine does not react as rapidly as uric acid with uric acid reagent, Folin and Marenzi found it necessary to add much more than that used by Folin and Looney in 1922. They also add the sulfite to the acid cystine solution, that is, before instead of after the carbonate, thereby cutting down the amount of 20% sulfite from 20 to 2 ml.; this makes the blank produced by sulfite practically negligible. The chief feature of this modification, however, is the substitution of the uric acid reagent, free from phenol, for that of Folin and Denis.

Reagents. *Marenzi Uric Acid Reagent, free from phenol.*[109] Dissolve in a Florence flask 100 g. of sodium tungstate in 200 ml. of water, add slowly, while shaking, and cooling, 20 ml. of 85% H_3PO_4; then pass H_2S into the mixture slowly for 20 minutes. At

the end of the first 3 or 4 minutes, add slowly 10 ml. additional of 85% H_3PO_4 without interrupting the hydrogen sulfide current. After 20 minutes, filter the solution, which is slightly acid to Congo red, returning the first portions of the filtrate to the paper if turbid. Transfer the clear greenish filtrate to a 1-liter separatory funnel, add with shaking 300 ml. (1.5 volumes) of ethanol. Draw off at once from the reddish or slightly greenish supernatant solution and the insoluble molybdenum sulfide beneath it the bluish heavy solution at the bottom, containing all the phosphotungstic acid, into a weighed 500-ml. flask, then add water until the weight of the contents is 300 g. Heat the solution over a micro burner for a few minutes until a paper moistened with lead acetate solution shows that the H_2S has been removed. Then cut down the flame, add 20 ml. of 85% H_3PO_4, at which point the ordinary (1 + 24) phosphotungstic acid is transformed to the active (1 + 18) phosphotungstic acid, that is, to the uric acid reagent. With a funnel and flask of cold water serving as a condenser in the neck of the 500-ml. flask, boil gently 1 hour, cut down flame, remove the condenser, filter, add a few drops of bromine, and boil to remove the blue color of the solution. Boil rapidly for a few minutes to remove the bromine, cover the flask, and cool under the tap. Place in a 1-liter beaker 25 g. of Li_2CO_3 and 50 ml. of H_3PO_4, after which add slowly 250 ml. of water and boil to remove the carbon dioxide. Cool and add to the concentrated uric acid reagent in the 500-ml. flask, then dilute to 1 liter.

Standard Cystine Solution, in 1.0 *N* sulfuric acid; 1 ml. = 1 mg. of cystine.

PROCESS. *Hydrolysis.* Place in a 300-ml. Kjeldahl flask 1 to 5 g. of dried protein, add 20 ml. of *6 N sulfuric acid* and 2 ml. of *butanol* (to prevent foaming), and reflux on the sand bath 18 to 20 hours. Remove the condenser and boil off the butanol. Dilute the hydrolyzate in a volumetric flask to 100 ml. and mix. Place 2 g. of *kaolin* in a 200-ml. flask, add the hydrolyzate, shake gently 3 to 5 minutes, and filter.

If desired, the kaolin can be added to the undiluted hydrolyzate in the Kjeldahl flask, then filtered, thoroughly washed, and diluted to 100 ml.

Color Formation. Transfer 1 to 5 ml. to a 100-ml. volumetric flask; add 2 ml. of *standard cystine solution* to another flask. To each add 2 ml. of freshly prepared *20% sodium sulfite solution* and let stand 1 minute. To the standard add 18 ml. of *20% sodium carbonate* solution and to the unknown add 18 ml. plus 0.5 ml. for each milliliter by which the hydrolyzate taken exceeds 2 ml. (example: if 4 ml. taken, use 19 ml. of carbonate). Add also 2 ml. of *20% lithium sulfate solution* and, with shaking, 8 ml. of *uric acid reagent.*

Color Comparison. After 3 to 5 minutes, dilute to volume and make the color comparison.

EXAMPLES. Casein 0.30, gliadin 2.19, edestin 1.35, zein 1.03, and egg albumin 1.22%.

III. Thomsett Modification.[110] Thomsett substitutes *sodium bicarbonate* for the carbonate and notes that differences in the concentration do not influence the results.

IV. Marenzi Micro Modification.[111] REAGENTS. See Folin and Marenzi Modification above.

Standard Cystine Solution, 0.1% in 0.2 *N* H_2SO_4. Dilute 10 ml. to 50 ml. with water for use.

PROCESS. *Hydrolysis.* See Folin and Looney Method above.

Color Formation. Place in test tubes or Nessler tubes, with 25-ml. graduation, suitable volumes of the *standard cystine solution* and of the unknown containing between 0.2 and 0.8 mg. of cystine. Add 0.2 ml. of *20% sodium sulfite solution,* shake for 1 minute, then add 0.2 ml. of *20% lithium sulfate solution* and 2 ml. of *20% sodium carbonate solution,* followed by continual shaking with 2

ml. of the *uric acid reagent.* After 3 to 4 minutes dilute to the 25-ml. mark with *2% sodium sulfite solution.*

Color Reading. Compare the known and unknown after 1 to 2 minutes.

EXAMPLES. By the Folin and Marenzi and the Marenzi methods respectively, the following results are given: casein 0.30 and 0.316; gliadin 2.19 and 2.22; edestin 1.37 and 1.35%.

V. Lugg Modification for Cystine and Cysteine.[112] After a study of the errors of the Folin and Looney method, Lugg (University of Adelaide) adopted the following reagents and procedure.

APPARATUS. *Colorimeter.*

REAGENTS. *Phosphotungstic Acid Reagent.* See Folin and Marenzi Modification above.

Sodium Acetate Solution, 4 *M.* Dissolve 544 g. of the salt in water and dilute to 1 liter.

Composite Citrate Buffer. Shake 105 g. of $H_3C_6H_5O_7 \cdot H_2O$ and 52.5 g. of NaOH with 500 ml. of water with constant cooling until dissolved; when cold, add a solution of 13.6 g. of $ZnCl_2$ dissolved in 20 ml. of water, then 26.8 g. of NH_4Cl dissolved in 200 ml. of water. Dilute to 1 liter.

Buffered Sulfite. Dissolve 9.5 g. of sodium meta-bisulfite ($Na_2S_2O_5$) in 70 ml. of water, add 15 ml. of 4 *M* sodium acetate solution, and dilute to 100 ml. The solution will keep for months.

Mercuric Chloride Solution, 0.1 *M.* Dissolve 2.72 g. of $HgCl_2$ in water and dilute to 100 ml.

Ferrous Salt Solution, $M/12Fe^{++}$. Dissolve 3.268 g. of Mohr's salt, $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, and 8.5 ml. of 1.0 *N* H_2SO_4 in water and dilute to 100 ml. It keeps well. Dilute 1 in 8, 1 in 9, 1 in 10, etc., before use.

Standard Cystine Solution, $M/240$ in 0.1 *N* HCl. Dissolve 0.5 g. of *l*-cystine in 50 ml. of 1.0 *N* HCl and dilute to 500 ml.; 1 ml. = 1 mg. of cystine.

PROCESS. A. CYSTEINE. *Color Formation.* Pipet 10 ml. of the *citrate buffer,* 2 ml. of the *phosphotungstic acid reagent,* and 2 ml. of *sodium acetate solution* into a 100-ml. flask in the order named and to the mixture add the chosen aliquot of the unknown cysteine solution.

Treat in like manner a solution of the standard, then add 1 ml. of the *buffered sulfite.*

Color Comparison. After 5 to 10 minutes, dilute the solutions and compare. The apparent cysteine content will be $x + a$ mg. in which x is the true amount and a will be zero in the absence of extraneous reducers. If these latter are present, prepare another unknown as before, except that 1 ml. of *mercuric chloride solution* is added prior to the unknown cysteine solution. Record the color developed, calculated as cysteine, as A mg. By trial in a series of calculations, determine the amount of ferrous salt solution needed to make the total coloration agree with the apparent $x + a$ mg. of cysteine. Prepare a new unknown containing *mercuric chloride* and unknown cysteine solutions, followed by the necessary amount of *ferrous salt solution,* and compare with a new standard containing the same amount of *ferrous salt solution* but none of the unknown cysteine solution. The contribution of the extraneous reducers to the color developed by the ferrous salt is found to be a' in terms of milligrams of cysteine, which may be different from A, but is nearly the same as a and hence x is found from $x + a - a'$.

B. CYSTINE. Prepare a *standard* and unknown in the same manner as the standard cystine plus sulfite solution used in estimating cysteine. The apparent cystine found will be $y + b$ mg., y being the true amount and b zero when extraneous reducers are absent. If such are present, prepare another unknown as before which contains *sulfite solution,* but add 1 ml. of *mercuric chloride solution* prior to the unknown cystine solution. The color will compare with b mg. of

cystine. By trial, determine the amount of ferrous salt solution necessary to match closely the original apparent $y + b$ mg. of cystine. Prepare a new unknown containing *mercuric chloride solution*, the unknown cystine solution, and *sulfite* and, after color development has proceeded 1 minute, add the necessary amount of *ferrous salt solution.* After the color development has continued for the remainder of the 5 to 10 minutes, dilute and compare with a new standard containing *mercuric chloride*, *sulfite*, and the same amount of *ferrous salt* but none of the unknown cystine solution. The increase in color formed by the extraneous reducers to that formed by the ferrous salt in the presence of sulfite corresponds with b' mg. of cystine. Identifying b' with b, y is found from $y + b - b'$.

C. CYSTEINE AND CYSTINE TOGETHER. Combine the procedures for the two amino acids as given above.

VI. Schöberl and Rambacher Modification.[113] After a study (Würzberg University) of the conditions for the complete reduction of 9-phosphotungstic acid (abbreviated to SWO_3) by cysteine, two reactions are postulated as taking place together as follows:

(1) $2R \cdot SH$ -

$$+ SWO_2$$

(2) $R \cdot SS \cdot R$

$$R \cdot SNa + R \cdot S \cdot SO_3Na$$

Only when the second reaction is complete is the color development at the peak. They determine the extinction coefficient (EK) in the Pulfrich photometer equipped with an S 72 filter. The calculation curve is plotted with molecular $R \cdot SH$ concentration from 1 to 10 as abscissas and EK (absorption) values from 0 to 2.6 as ordinates. Thioglycolic acid and cysteine are proportional in their action, obeying Beer's law. A definite temperature (20°) is essential.

Blankenstein Nitroprusside Colorimetric Method.[114] The method was devised at Landestad, Aachen.

APPARATUS. *Colorimeter.*

REAGENT. *Standard Cystine Solution*, 20 mg. per 100 ml. Mix 20 ml. of 0.1% cystine solution in 1.0 N H_2SO_4 with 30 ml. of 20 volume per cent H_2SO_4 and 50 ml. of N H_2SO_4.

PROCESS. *Hydrolysis.* Reflux 0.5 to 1 g. of the protein with 15 to 20 ml. of *20% sulfuric acid* for 12 hours. Shake the hydrolyzate with *bolus alba*, filter into a 50-ml. volumetric flask, and wash with *1.0 N sulfuric acid* up to volume.

Color Formation. Mix 10 ml. each of the hydrolyzate and the *standard cystine solution* with 5 ml. of *0.02 N potassium cyanide solution* and 6 ml. of *ammonium hydroxide* in a 25-ml. volumetric flask, cover with a funnel, and heat for 15 minutes in a boiling water bath, then cool under the tap and make up to volume with *ammonium hydroxide.* Add 6 to 8 drops of freshly prepared *5% sodium nitroprusside solution.*

Color Comparison. Match the colors of standard and unknown, setting the standard at 5 mm.

CALCULATION. Ten milliliters of the standard contains 0.002 g. of cystine and 10 ml. of the hydrolyzate (if 0.5 g. were hydrolyzed) represents 0.1 g. of the protein, then the percentage of cystine (P) in the protein is obtained from the following equation:

$$P = \frac{100 \times 0.002 \times 5}{0.1 \times R} = \frac{1}{0.1 \times R}$$

in which R is the reading of the unknown.

Zittle and O'Dell Cuprous Oxide Gravimetric Method.[115] Reduction and precipitation of the cystine as mercaptide is the salient feature and this, taken in conjunction with the eventual weighing of the sulfur as barium sulfate, stamps the method as novel throughout. The authors (University of Pennsylvania) regard the Sullivan colorimet-

ric procedure for cysteine more accurate and convenient than the gravimetric.

APPARATUS. *Colorimeter.*

REAGENTS. *Cuprous Oxide Suspension.* Add a solution of 7 g. of $CuSO_4 \cdot 5H_2O$ in 100 ml. of water to 100 ml. of a solution of 170 g. of sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$), 520 ml. of water, and 80 ml. of saturated NaOH solution. Heat to boiling, add 1 g. of dextrose in 200 ml. of water and, after boiling several minutes, wash the precipitate with hot water by decantation (about four times) until the washings are neutral, then suspend in 100 ml. of water. The suspension contains about 25 mg. of cuprous oxide per milliliter.

Sodium Acetate Solution. Dissolve 110 g. of $NaC_2H_3O_2 \cdot 3H_2O$ in 100 ml. of water.

Fusion Accelerator. Add 600 mg. of $KClO_4$ to 100 mg. of benzoic acid.

Acid Potassium Chloride Reagent. A 25% KCl solution in 1% HCl.

Sodium Sulfide Solution, 10% in 0.5 *N* NaOH.

Standard Cystine Solution, containing 0.80 mg. per ml.

PROCESS. *Hydrolysis.* Reflux an amount of the protein that will yield about 24 mg. of cysteine with 50 ml. of *1 + 1 hydrochloric acid* for 18 hours and evaporate the hydrolyzate to complete dryness *in vacuo.* Take up in water and 2 ml. of *1.0 N hydrochloric acid,* filter into a 100-ml. volumetric flask, dilute to the mark, and remove an aliquot of 30 ml. for mercaptide precipitation and of 10 ml. for the Sullivan colorimetric comparison.

Cuprous Oxide Precipitation of Cysteine. Heat to boiling the aliquot in a 50-ml. centrifuge tube and add eight times the theoretical amount of *cuprous oxide suspension* with stirring, noting that at first the cuprous oxide dissolves, then the greenish gray mercaptide begins to form. About a minute after the last of the reagent is added, pour 0.5 ml. of *sodium acetate solution* into the mixture with stirring, thus bringing the *p*H to about 4.0, the point of minimum solubility of the mercaptide causing its flocculation. Let stand 40 minutes at room temperature for complete precipitation, centrifuge 5 minutes, and decant carefully the clear supernatant liquid. Wash the precipitate once by suspending in 25 ml. of *ethanol* for several minutes, centrifuge, and decant.

A. Gravimetric Procedure. Dry the precipitate from the 30-ml. aliquot in the centrifuge tube at 100° for 15 minutes. Remove the bulk mechanically to a Parr peroxide bomb cup and the remainder by the use of repeated small portions of the *fusion accelerator.* Conduct the fusion, dissolve the melt, and precipitate the barium sulfate as directed in the manual supplied with the Parr bomb. Collect the precipitate on a weighed filter crucible of suitable porosity, ignite at dull redness 1 hour in the electric furnace, cool, weigh, and from the weight calculate the cystine.

B. Sullivan Colorimetric Procedure. Dissolve the ethanol-washed precipitate of the 10-ml. aliquot, after draining, in 5 ml. of *acid potassium chloride reagent,* and transfer to a 15-ml. graduated centrifuge tube, rinsing with water to about 11 ml. Precipitate the copper in one step by adding with stirring 10 drops of *pyridine,* followed by 0.5 ml. of *10% potassium thiocyanate solution.* Dilute to the 15-ml. mark, centrifuge to cause the dense precipitate to deposit, and pipet 5-ml. aliquots of the supernatant liquid into 15 x 2.5 cm. test tubes with 20-ml. graduation marks. Add 0.5 ml. of *5 N sodium hydroxide solution* and develop the color by the Rossouw and Wilken-Jorden procedure.[116] Allow the *0.5% sodium naphthoquinone sulfonate solution* to react 20 seconds before adding 5 ml. of the *alkaline sodium sulfite solution.* After the addition of the final reagent, dilute to 20 ml., transfer to the colorimeter tube, and allow to stand 3 to 4 minutes before reading.

Color Comparison. Match the unknown with the known (standard containing 0.8 mg.

of cystine per milliliter) treated in like manner.

Okuda Iodide-Iodate Volumetric Method.[117] Two methods have been proposed by Okuda, one employing sodium bromide and titrating with sodium bromate, the other employing potassium iodide and titrating with potassium iodate. Although the same in general principle, the first is simple but accurate only in the absence of other amino acids, hence is admittedly impracticable for general use.

The iodide-iodate method is claimed to be applicable to all protein hydrolyzates. The reactions are as follows:

(1)

$$6KCl + 3H_2O + 3I_2$$

(2) $\underset{\text{Cystine}}{C_6H_{12}O_4N_2S_2} + 10I + 6H_2O \rightarrow$

$$\underset{\text{Cysteic acid}}{2HOOC \cdot CH(NH_2)CH_2 \cdot SO_2 \cdot OH} + 10HI$$

The directions given below apply to the determination of total cystine, that is, cystine (which predominates) plus cysteine, the former being reduced to the latter by zinc dust. If the content of each separately is desired (this is unusual), cysteine may be determined by omitting the reduction with zinc and cystine may be obtained by difference.

Reagent. *Standard Potassium Iodate Solution,* 0.03 *M* in 2% HCl. Dissolve 2.14 g. KIO_3 in 3 liters of 2% HCl. Standardize against the standard cystine solution.

Standard. *Cystine Solution.* Dissolve 1 g. of cystine in 50 ml. of 5% HCl, add a few decigrams of zinc dust, allow to react at room temperature for 30 minutes with shaking, filter, wash, and make up to 100 ml. with water. Immediately transfer 1 ml. of the filtrate to a test tube and mix with exactly 19 ml. of 2% HCl, then add 5 ml. each of 5% KI and 4% HCl, and titrate 1-ml. portions at different temperatures with 0.03 *M* KIO_3 to a faint yellow color that remains permanent for 1 minute; 1 ml. of iodate solution = 0.0101 g. of cystine.

Prepare a graph showing the milliliters of standard iodate solution corresponding to 1 mg. of cystine at different temperatures.

Process. *Hydrolysis.* Reflux 1 to 10 g. of the sample with 3 times its weight of *hydrochloric acid,* at first over a boiling water bath and later for 20 hours over the sand bath. Evaporate under reduced pressure to remove the excess over 6 g. of *hydrochloric acid,* then add a little water, decolorize by heating for 30 minutes with *charcoal,* filter, and wash into a 100-ml. volumetric flask.

Reduction to Cysteine. To the cooled filtrate add a few decigrams of *zinc dust* and allow to reduce at room temperature for 30 minutes with occasional shaking, after which filter, make up to 100 ml., and mix. Titrate an aliquot of 1 ml. to determine its acidity, then adjust the remainder to exactly 2% hydrochloric acid.

Titration. Transfer a measured amount of the solution to a clean dry flask or large test tube, dilute with exactly *2% hydrochloric acid* to a volume of 20 ml., then add 5 ml. each of *5% potassium iodide solution* and *4% hydrochloric acid,* and titrate with *standard potassium iodate solution,* preferably at 17.5°, until a permanent yellow color appears. Record the exact temperature if other than 17.5°, as well as the volume of standard solution.

Calculation. Calculate the weight of total cystine, correcting for the temperature as indicated on the graph. If the temperature is 17.5°, calculate the total cystine content (*C*) by the formula

$$C = \frac{0.0101 \times K}{4.65}$$

in which *K* represents the milliliters of potassium iodate solution required for the titration.

To obtain the ready-formed cysteine,

make a separate determination on an aliquot of the solution of the hydrolyzate, omitting the reduction with zinc dust. Calculate the cystine content by the above formula.

To obtain the cystine content, subtract the cysteine content from the content of total cystine.

Baernstein Iodine-Hydrazine Gasometric Method.[118] The salient feature of the method (University of Wisconsin) is the oxidation of cysteine to cystine by iodine in acid solution and the determination of the excess by measurement of the gas resulting from its reaction with hydrazine.

Apparatus. *Van Slyke and Neill Manometer.*

Reagents. *Iodine Reagent.* Dissolve 30 g. of KI in 100 ml. of water, add 10 g. of iodine crystals, and dilute to 4 liters when the iodine has dissolved.

Hydrazine Solution. Saturate distilled water with $H_2N \cdot NH_2 \cdot H_2SO_4$ and mix with an equal volume of 40% NaOH.

Process. *Hydrolysis.* Follow an approved procedure.

A. Preformed Cysteine. *Iodine Treatment.* Pipet 2 ml. of a solution containing 1 to 4 mg. of cysteine in *1 to 8 N hydrochloric acid* into a 15-ml. centrifuge tube. Add 1 ml. of *hydrochloric acid* and 2 ml. of *iodine reagent,* mix by inverting several times, and allow to come to room temperature; then shake vigorously to saturate with air.

Hydrazine Treatment. Draw 1 ml. of the mixture into a Van Slyke and Neill glass stopcock pipet, provided with a device for preventing the entrance of the acid into the mouth, and introduce it under mercury into the chamber of the manometric apparatus previously charged with 2 ml. of *hydrazine solution.* Lower the leveling bulb and while the mixture is descending in the chamber seal the upper cock with mercury. Rinse the chamber by raising and lowering the bulb several times and evacuate to the 50-ml. mark, then shake for 2 minutes.

Reading. Read the pressure of the gas (p_1) at a volume of 0.5 ml. This gas consists of air from the reagents to the extent of about 100 mm. pressure, the remainder being nitrogen from the hydrazine reaction.

Blank. At the same temperature analyze a blank containing 2 ml. of *hydrochloric acid* instead of cysteine solution, giving p_0, which includes the air in the reagents and nitrogen liberated by all the iodine employed.

Subtract p_1 from p_0, thus obtaining the value Δp which is strictly proportional to the amount of cysteine in the sample.

B. Cystine. Place 5 ml. of a solution containing less than 10 mg. of cystine in *1 to 8 N hydrochloric acid* in a 50-ml. flask and add a few grams of *Devarda alloy.* Close with a stopper provided with a capillary tube 5 cm. long, let stand 10 to 15 minutes with occasional shaking, and filter from the undissolved alloy.

Iodine Treatment. Proceed as described under A. Treat a blank in the same manner as the actual analysis.

Calculation. Since the difference in pressure (Δp) between the blank and the actual analysis is proportional to the amount of cysteine present, factors may be calculated for converting millimeters of pressure into milligrams of cysteine or cystine. From a large number of determinations Baernstein found that $\Delta p = 48.1 \pm 0.7$ mm. at 25°, which is equivalent to 3.85 mg. of cysteine or 3.79 mg. of cystine.

Factors (F) for various temperatures which appear in the table below are based on the following equation:

$$F = \frac{1}{P} \times \frac{273 + 25}{273 + t}$$

in which $P = \Delta p$ per mg. of cysteine or cystine = 12.62 mm. or 12.84 mm. respectively.

C. Cystine in Proteins. Hydrolyze 1 g. of the protein by refluxing for 15 hours with 20 ml. of *20% hydrochloric acid.* Wash the

t	Cysteine	Cystine	t	Cysteine	Cystine
°C.			°C.		
20	0.0813	0.0799	26	0.0797	0.0786
21	.0811	.0797	27	.0794	.0779
22	.0808	.0794	28	.0792	.0778
23	.0805	.0792	29	.0787	.0776
24	.0803	.0789	30	.0783	.0773
25	.0801	.0787			

hydrolyzed protein into a 25-ml. volumetric flask with 5 ml. of *hydrochloric acid*. Treat an aliquot of 10 ml. of the hydrolyzate with *Devarda alloy* as described above and use 2 ml. of the solution for the analysis.

EXAMPLES. Egg white 2.07, egg albumin 1.58, gelatin 0, edestin 1.75, and casein 0.94% of cystine.

Fujita and Numata Dimethyl-Phenylenediamine Colorimetric Method.[119] APPARATUS. *Pulfrich Photometer*, with 5 mm. cell and filter S 61.

REAGENTS. *Diamine Reagent*. Dissolve 50 mg. of dimethyl-*p*-phenylenediamine hydrochloride in 100 ml. of 4 *N* H_2SO_4. The solution keeps at least 1 week.

Ferric Salt Reagent. Dissolve 10 g. of $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ in 100 ml. of 1.0 *N* H_2SO_4. The solution keeps a long time.

PROCESS. A. CYSTEINE. *Preparation of Protein Filtrate*. Hydrolyze or otherwise prepare a solution of the protein or protein-containing material such as liver or kidney. Treat the solution with *2% meta-phosphoric acid* and filter to remove the albumin.

Color Formation. To 2 ml. of the filtrate contained in a test tube with a mark showing 4.2 ml. or a graduated cylinder, add 2 ml. of the *diamine reagent* and 0.2 ml. of the *ferric salt reagent*. Mix and heat the mixture 40 minutes in a boiling water bath. Cool under the tap, fill again to the mark, centrifuge, and decant the clear liquid.

Color Measurement. Determine the extinction coefficient of the dark red-violet color, specific for cysteine, in the Pulfrich photometer using filter S 61 and the 5-mm. cell.

CALCULATION. Obtain the milligrams of cysteine (X) per 100 ml. in the solution (unknown) from the formula

$$X = \qquad - E_0) \times V$$

in which E and E_0 are the extinction coefficients of the unknown and the blank, and V is the concentration (dilution).

B. TOTAL CYSTEINE. *Preparation of Protein Filtrate*. As under A.

Mercuric Acetate Treatment. To 5 ml. of the deproteinized filtrate, add 4 ml. of *1% mercuric acetate solution* and 0.5 ml. of *2 N hydrochloric acid*. Mix, add 0.5 ml. of *50% sodium acetate solution*, saturate with *hydrogen sulfide gas*, and allow to stand overnight in an atmosphere of *hydrogen sulfide*. Filter, add to 8 ml. of the filtrate 0.3 ml. of *2 N hydrochloric acid*, remove the hydrogen sulfide *in vacuo*, replace the water lost by evaporation, and filter through a dry paper.

Color Formation. To 2 ml. of the filtrate add 2 ml. of the *diamine reagent* and 0.2 ml. of the *ferric salt reagent*, mix, and heat 40 minutes in a boiling water bath. Cool under the tap and fill to the 4.2-ml. mark. Allow to stand 10 to 30 minutes, centrifuge and decant the clear supernatant liquid.

Color Measurement. Determine the extinction coefficient as above.

CALCULATION. As above.

Sullivan Sodium Naphthoquinone Sulfonate Colorimetric Method.[120] In announcing his test, Sullivan (U. S. Public Health Service) states that cysteine is the only amino acid that gives immediately a reddish orange to dark brown or red color with sodium 1,2-naphthoquinone-4-sulfonate, in the presence of an alkali and sodium sulfite (Na_2SO_3), sodium thiosulfate ($Na_2S_2O_3$), or other reducing agent, that is not discharged

by an alkaline solution of sodium hydrosulfite ($Na_2S_2O_4$). Cystine is gradually reduced by the sulfite and gives the reaction slowly.

If sodium cyanide is first added to a cystine solution, followed by the naphthoquinone sulfonate and the other reagents, the characteristic cysteine color is formed, owing to the reduction of cystine to cysteine.

The details of the quantitative method here given apply to the determination of cystine plus cysteine (total cysteine). To obtain the cysteine content only, omit the first addition of the sodium cyanide; to obtain the cystine content only, subtract the content of cysteine from that of the joint substances.

REAGENTS. *Sodium Sulfite Solution,* 10% in 0.5 *N* sodium hydroxide.

Sodium Hydrosulfite Solution ($Na_2S_2O_4$), 2% in 0.5 *N* sodium hydroxide.

Standard Cystine Solution. Dissolve 10 mg. in 100 ml. of 0.1 *N* HCl.

PROCESS. *Hydrolysis.* Reflux 5 g. of the protein with 15 to 20 ml. of *20% hydrochloric acid,* heating in a Crisco bath for 6 hours after the bath reaches 125° (inside about 106°). Transfer the black mixture to a 100-ml. beaker, rinse with water, and adjust to 10% acid with *hydrochloric acid.* Decolorize by warming with 0.4 g. of *carboraffin.* Filter on a small Büchner funnel, extract the residue on the paper with 15 ml. of hot *1.0 N hydrochloric acid,* and wash with 15 ml. of the cold *1.0 N acid.* Adjust the combined filtrate and washings with *5 N sodium hydroxide solution* to pH 3.5 (yellow to *thymol blue,* green-blue to *bromophenol blue*), and dilute to 100 ml. in a volumetric flask with *0.1 N hydrochloric acid.*

One gram of the sample may be hydrolyzed in the same manner by using proportionately smaller amounts of reagents and diluting finally to 25 ml.

Color Formation. Bring 5 ml. of the neutralized hydrolyzate, also an aliquot of *standard cystine solution,* to a definite volume and a temperature not under 20°, add 2 ml. of *5% sodium cyanide solution,* and mix; then after 10 minutes add 1 ml. of *0.5% 1,2-naphthoquinone-4-sodium sulfonate solution* and mix. After 10 seconds add 5 ml. of the *alkaline 10% sodium sulfite solution,* mix, let stand 30 minutes, then add 2 ml. of 5 *N sodium hydroxide solution* and mix. Finally add 1 ml. of the *alkaline 2% sodium hydrosulfite solution.*

The addition of the last reagent changes the color from brown-red to vivid red, with a tendency to orange for solutions weaker than 100 mg. per kilo of cystine. The colors produced by other substances in the earlier stages are changed to yellow by the hydrosulfite in the absence of cystine and cysteine.

Color Reading. Compare the color of the unknown with that of the solution of pure cystine, both treated in the same manner.

I. Sullivan and Hess Modification for Cysteine in the Presence of Glutathione.[121] The authors, pursuant to criticism by Meldrum and Dixon, found that when the glutathione-cysteine ratio is 9 : 1 and even 18 : 1, the procedure as given above yields satisfactory results, but for higher ratios more of the naphthoquinone sulfonate must be used, thus:

To 5 ml. of a solution containing glutathione and cysteine in the ratio of 36 : 1 and a standard solution containing 1 mg. of cystine in 5 ml. of 0.1 *N hydrochloric acid,* add 1 ml. of *1% sodium cyanide solution,* 1 ml. of *1% sodium naphthoquinone sulfonate solution,* and 5 ml. of the *alkaline 10% sodium sulfite solution.* After allowing to stand 30 minutes, add 1 ml. of the *alkaline 2% sodium hydrosulfite solution.*

For a solution with a glutathione-cystine ratio of 100 : 1, use 2 ml. of *1% sodium naphthoquinone sulfonate solution* and 1 ml. of *alkaline 1.0 N sodium hydrosulfite solution,* but in other respects proceed as with the 36 : 1 solution.

II. Sullivan and Hess Modification Employing Hydrolysis in the Presence of Titanous Chloride.[122] The procedure was elaborated at Georgetown University.

REAGENTS. *Sodium Cyanide Solution,* 1% in 0.8 *N* NaOH.

Standard Cysteine Solution. Dissolve 1.3 mg. of cysteine chloride (1 mg. of cysteine) in 100 ml. of 0.1 *N* HCl.

Other reagents as in the original method.

PROCESS. *Hydrolysis.* Place 1 g. of the protein (such as grain curd casein) together with 5 ml. of *20% hydrochloric acid* and 1 ml. of *20% titanous trichloride solution* in a small acetylation flask fitted with a reflux condenser. Heat in a boiling oil bath at 125° for 1 to 2 hours. Pour into a small beaker and rinse the flask with 5 ml. of water. Cool, neutralize to about *p*H 6 with *5 N sodium hydroxide solution* added dropwise with stirring. Filter by suction the solution from the precipitate of blue gelatinous titanous hydroxide (TiO_3H_3), wash with 5 ml. of water, and adjust the filtrate to *p*H 3.5 with *hydrochloric acid,* then dilute to 35 ml. with *0.1 N hydrochloric acid.*

A. CYSTEINE. *Color Formation.* To 5 ml. of the hydrolyzate add 1 ml. of the *naphthoquinone reagent,* shake 10 minutes, then add 5 ml. of *10% sodium sulfate reagent,* followed by 1 ml. of the *alkaline sodium cyanide solution,* mix, let stand 30 minutes, then add 2 ml. of *5 N sodium hydroxide solution* and 1 ml. of *sodium hyposulfite reagent.*

Color Reading. Compare the color of the unknown with 5 ml. of the cysteine standard solution similarly treated.

B. CYSTINE. *Color Formation.* To 5 ml. of the solution, add 2 ml. of *5% aqueous sodium cyanide solution* (or better 2 ml. of *5% sodium cyanide in 1.0 N sodium hydroxide solution*), mix well, and let stand 10 minutes with the temperature not lower than 20°. Add *naphthoquinone solution, sodium sulfite solution,* etc., as in the cysteine procedure, but omit the 1% sodium cyanide solution.

Color Comparison. Match the solution against 5 ml. of a suitable cystine standard similarly treated.

Vassel Aminodimethylaniline Colorimetric Method.[123] The action of cystine with the reagent in the presence of iron ions, first observed by Fleming,[124] is the basis of a method developed at the laboratory of the American Cyanamide Company. The advantage of simplicity over the Fujita and Numata method is claimed.

APPARATUS. *Hardy Spectrophotometer.*

REAGENTS. *Acid.* One having the same composition and normality as the protein hydrolyzate.

Dye Solution. Dissolve 35 mg. of *p*-aminodimethylaniline monohydrochloride (Eastman No. 492) in 100 ml. of 6 *N* H_2SO_4. Keep in the dark in a refrigerator at 5° and make fresh every 10 to 14 days.

Ferric Ammonium Sulfate Solution. Dissolve 20 g. of $FeNH_4(SO_4)_2 \cdot 12H_2O$ (reagent grade) and dilute to 100 ml. with 1.0 *N* H_2SO_4.

Zinc Dust. Mallinckrodt reagent grade, containing no impurity insoluble in 1.0 *N* H_2SO_4 on heating.

Standard Cysteine Solution. Dissolve in acid of the same normality as the protein hydrolysis mixture.

PROCESS. A. CYSTINE. *Color Formation.* Pipet 1 ml. of the solution or hydrolyzate, containing 0.01 to 0.20 mg. of cystine, into an 18 x 150 mm. Pyrex test tube. If less than 1 ml. is used, add the *acid* to a final volume of 1 ml. Then add in the order named 3 ml. of the *dye solution,* exactly 165 mg. of *zinc dust,* and, after 2 to 4 minutes, 2 ml. of *ferric ammonium sulfate solution.* Allow the reducing action of the zinc dust to proceed for 45 minutes with occasional mixing to counteract the tendency of the dust to float, then add 3 ml. additional of the *ferric ammonium sulfate solution.* Stopper lightly, immerse in a boiling water bath, and hold there for 45 minutes. To insure complete solution of the zinc dust, carefully wet the

walls of the test tube twice with the hot solution after heating 5 to 10 minutes. After heating, cool the mixture in a cold water bath, thus changing the greenish blue color to a deep reddish blue. Transfer the liquid to a 25-ml. volumetric flask and make up to volume with water.

Color Measurement. Determine the color intensity in a 1-cm. cell at its maximum absorption band, 5750 to 5800 Å in the Hardy ectrophotometer. The absorption at 5800 is constant for 30 minutes, after which it increases slowly.

Prepare a cystine curve, using a solution approximating in acid strength as closely as possible that of the solution of the unknown, showing 0.01 to 0.20 mg. by 0.01 increments of cystine, between which limits Beer's law is valid, as abscissas and extinction coefficients $\left(-\log \frac{T}{\;}\right)$ from 0.100 to 0.500 by 0.025.

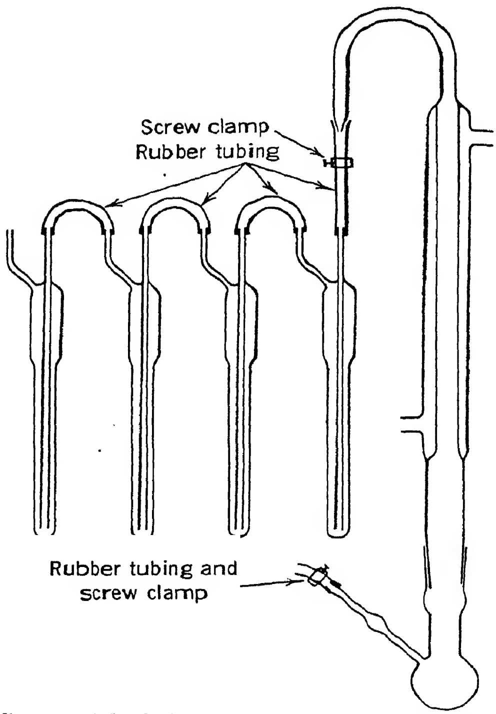
B. CYSTEINE. The procedure below may be followed even in the presence of cystine.

Color Formation. Add 165 mg. of *zinc dust* to a mixture of 3 ml. of the *dye solution* and 2 ml. of the *ferric ammonium sulfate solution* in an 18 x 150 mm. Pyrex test tube. Allow the zinc dust to react with the acid for 10 minutes at room temperature, then for complete solution immerse in boiling water for 15 to 35 minutes, taking care that no zinc escapes solution. Cool rapidly, then add 1 ml. of the solution of the unknown, followed immediately by 3 ml. of *ferric ammonium sulfate solution.* Heat in a boiling water bath for 45 minutes, then proceed as in the determination of cystine.

EXAMPLES. Casein, cystine 0.32; lactalbumin, cysteine 0.52, cysteine plus cystine 2.61; and edestin, cystine 1.18%.

METHIONINE

Baernstein Homocysteine Volumetric Method.[125] The method (University of Wisconsin) depends on the demethylation of methionine and oxidation of the resulting homocysteine with sodium tetrathionate. In an earlier method,[126] a similar apparatus train was employed, but the methionine was



Courtesy of the Author and J. Biol. Chem. 1936, 115, 25

FIG. 55. Baernstein Methionine Apparatus.

determined only by the titration of the volatile iodide.

APPARATUS. *Digestion Flask, Condenser,* and 4 *Scrubbers* are clearly shown in Fig. 55.

REAGENTS. *Hydriodic Acid-Phosphate Reagent:* 57% HI containing 1% of KH_2PO_4.

Sodium Tetrathionate Solution. Prepare from 0.1 *N* potassium biniodate and 0.1 *N* $Na_2S_2O_3$ with the aid of a little KI and HCl.

PROCESS. *Naphtha Extraction.* Treat the protein for 24 hours with *naphtha* in a contin-

uous extractor and dry in a vacuum desiccator over phosphoric acid.

Hydrolysis. Weigh 0.5 g. of the extracted protein into the digestion flask, add 10 ml. of *hydriodic acid-phosphate reagent* and a small boiling chip, and connect with the condenser held at 50 to 60°. In scrubber 1 place *20% cadmium chloride solution* and *20% barium chloride solution*, in scrubber 2 *saturated mercuric chloride solution*, and in scrubbers 3 and 4 *glacial acetic acid* with *10% potassium acetate solution* and 6 drops of *bromine* per 10 ml.

With the upper pinchcock open and the lower one closed, light the micro burner. When the bubbling, which begins at once, stops and, while boiling quietly, the iodine first formed is entirely reduced by the hyposulfite, open the lower cock, thus admitting *nitrogen*, and adjust the upper one to maintain a bubbling rate that can be counted. After the boiling and aeration have continued for 6 hours, disconnect the scrubbers and drain the condenser flask, then connect with a second cooled vertical condenser and concentrate the digest to about 3 ml., avoiding burning.

Dilution of Digest. Disconnect the flask containing the digest, add 2 or 3 crystals of *monopotassium phosphate*, and boil the mixture for 1 minute to remove iodine; then transfer to a 25-ml. volumetric flask, rinsing with *4% hydrochloric acid saturated with nitrogen.* Stopper tightly, cool under the tap, and make up to the mark.

I. Analysis of the Digest (Homocysteine Method). Measure two 10-ml. aliquots into 50-ml. Erlenmeyer flasks and remove air by suction. Disconnect the first flask and add a slight excess of *0.02 N potassium biiodate*, 2 ml. being required for every 10 mg. of cystine in 25 ml. of the digest. Then add a few drops of *starch solution* and titrate the excess of iodine with *0.02 N thiosulfate solution.*

Treat a blank digest without protein in like manner to determine the available iodine. From the iodine consumed, the amount of cystine may be calculated. Add 2 ml. of *sodium tetrathionate solution* and deaerate by suction through a three-way stopcock and rubber stopper (not shown). After the air has been removed, connect a buret containing *ammonium hydroxide* to the side tube of the stopcock and allow 3 ml. to enter; then evacuate the flask and close the stopcock, taking care to avoid loss by foaming.

Titration. After 15 minutes remove the flask, acidify with 10 ml. of *10% hydrochloric acid*, and titrate with *0.02 N potassium biiodate solution.* It is well to have only a small excess of tetrathionate and a minimum of ammonium hydrate, otherwise decomposition of the tetrathionate by alkali will cause a high blank.

Calculation. From the quantity of iodine consumed by the thiosulfate, which was formed in the reduction of tetrathionate by homocysteine, calculate the quantity of methionine.

II. Titration of the Volatile Iodide. Transfer the contents of scrubbers 3 and 4 to a 100-ml. volumetric flask containing 5 ml. of *25% sodium acetate solution*, rinse, and add a small excess of *formic acid* to reduce the excess of bromine. Centrifuge, dilute to the mark, pipet a 25-ml. aliquot into a beaker containing a little *potassium iodide solution*, and a few drops of *10% sulfuric acid*, then titrate the iodine with 0.02 *N thiosulfate solution.*

Calculation. Obtain the amount of methionine from the iodine equivalent of the thiosulfate required.

Examples. The following amounts of methionine were found by the volatile iodide and the homocysteine methods respectively: lactalbumin 2.45 and 2.32, casein 3.31 and 3.10, edestin 2.38 and 2.20, ovalbumin 5.07 and 4.49, ovomucoid 1.43 and 1.38, vitellin 2.70 and 2.60, and zein 2.58 and 2.46%. The

lower results by the homocysteine method are believed to be more nearly correct.

Beach and Teague Sulfur-Difference Gravimetric Method.[127] As developed at the Children's Fund of Michigan Laboratory, Detroit, the sulfur of cystine plus methionine is determined in one of two aliquots of a hydrolyzate and the sulfur of cystine in the other. The methionine content is calculated by multiplying the sulfur content obtained by difference by the proper factor. In a solution containing cysteine and homocysteine-thiolactone, only cysteine reacts with cuprous oxide to form a precipitate of an insoluble cuprous mercaptide. If, however, the ring is first opened by alkali, mercaptides of both cysteine and homocysteine may be precipitated together.

REAGENTS. *Cuprous Oxide,* bright red, containing a minimum amount of sulfur: Baker's c.p. Cu_2O, red powder, wet by shaking with water and ground to a thorough suspension, is satisfactory.

Citrate-Acetate Buffer Solution. Dissolve 12 g. of $Na_3C_6H_5O_7 \cdot 2H_2O$ and 15 g. of $H_3C_6H_5O_7 \cdot H_2O$ in 200 ml. of water and add 20 ml. of glacial acetic acid. Dilute this stock solution to 10 volumes for use.

PROCESS. *Hydrolysis.* Weigh a charge (usually 0.5 to 1.0 g.), containing about 6 mg. of methionine, into a 100-ml. round-bottom flask, add 25 ml. of *concentrated hydriodic acid,* and reflux 18 hours. Cool, connect with a vacuum still, and evaporate the hydrolyzate at about 40° under reduced pressure to 0.5 ml.

Removal of Iodine. Repeat the distillation several times with addition of *2.5% hydrochloric acid* to remove most of the hydriodic acid, transfer to a 250-ml. centrifuge bottle, and dilute to 50 ml. Add to the light tan-colored solution *moist silver chloride* in excess (about 2 g.), and shake vigorously. As the liquid turns a darker brown, continue the addition to insure complete removal of the hydriodic acid and separate the precipitate by centrifuging. Decant the supernatant liquid through a filter, wash with two 50-ml. portions of water by centrifuging, and evaporate *in vacuo* the combined solution and washings to a sirup of about 2 ml. Transfer the sirup with water to a 50-ml. volumetric flask, dilute to the mark, and pipet a 25-ml. aliquot (B) into a glass-stoppered centrifuge tube. Wash the pipet back into the volumetric flask with a little water (A).

A. CYSTINE (MODIFIED GRAFF, MACULLA, AND GRAFF METHOD).[128] *Reduction of Cystine to Cysteine.* To the 25-ml. aliquot (A) in the volumetric flask, add 1 ml. of *20% hydrochloric acid* and 300 mg. of *zinc dust.* Allow to stand at room temperature for 2 hours, then remove the zinc by filtration. Add to the filtrate *saturated sodium acetate solution* to pH 4 to 5 with rapid mechanical stirring.

Copper Precipitation. Continuing the stirring, add dropwise to the solution a suspension of finely divided *bright red cuprous oxide.* Continue the addition until the flocculant light gray precipitate of cysteine cuprous mercaptide acquires a red color with a small excess of the reagent; then stir for about 30 seconds to complete the reaction. Centrifuge, decant, wash three times with 30 ml. of *citrate-acetate buffer solution* to remove sulfur-containing contaminants, and determine the sulfur by the Micro Gravimetric Method as directed below.

B. CYSTINE PLUS METHIONINE. *Opening of Thiolactone Ring.* Neutralize the solution in the centrifuge tube (B) with *5 N sodium hydroxide solution* and add 1 ml. in excess. Allow to stand for 15 minutes, thus opening the ring without destroying the cystine.

Reduction. Acidify by adding 2 ml. of *20% hydrochloric acid,* then add 300 mg. of *zinc dust,* stopper loosely, and let stand overnight at room temperature. In the morning, heat for 2 hours in a steam bath without further adjustment of the pH.

Copper Precipitation. Decant quickly

from the excess of zinc into a centrifuge tube containing a few drops of *cuprous oxide suspension*. Without delay wash the zinc remaining in the tube with water and decant through a small filter. Stir rapidly for 15 seconds the combined solution and washings in the centrifuge tube together with an excess of *cuprous oxide suspension* at 60°, stopper immediately, and centrifuge 1 minute. Quickly remove the supernatant liquid by decantation. The precipitate is a mixture of cuprous mercaptides of cysteine and homocysteine. Omit the washing with the buffer solution, since contamination with sulfur is unlikely.

SULFUR DETERMINATION (MICRO MODIFICATION OF THE DENIS METHOD).[129] Dissolve the precipitate in 1.5 ml. of *nitric acid* and proceed as in the Denis method as follows. Perform the combustion with 1 ml. of *Denis reagent*, dissolve the mixture in 5 ml. of *10% hydrochloric acid*, filter, and precipitate the barium sulfate from a volume not exceeding 40 ml. Filter through the sintered Pyrex glass micro crucible prepared by cutting a filter stick of finest porosity 5 mm. below and 30 mm. above the sintered glass insert.

CALCULATION. Correct the weight of barium sulfate for losses by multiplying by the factor 1.11; 1 mg. of barium sulfate = 0.515 mg. of cystine or 0.709 mg. of methionine (corrected).

EXAMPLES. Edestin 2.30, arachin 0.57, casein 3.12, lactalbumin 2.98, beef muscle 3.21, gelatin 0.81, and egg white 4.09%.

PHENYLALANINE

Kollmann Dichromate-Benzoic Acid Gravimetric Method.[130] The phenylalanine is oxidized in an acid medium with the formation of benzoic acid (Vienna University).

APPARATUS. *Extractors* for fat and for fatty acids.

PROCESS. *Extraction of Fat.* Weigh 25 (casein) to 50 (fibrin) g. of the protein into a fat extractor, extract with *anhydrous ether* in the usual manner, and determine the fat and weight of dry fat-free substance in the charge.

Hydrolysis. Reflux the defatted material for 30 hours with *25% sulfuric acid,* using 10 ml. for each gram of pure protein.

Fatty Acids Extraction. Divide the hydrolyzed liquid into two portions, if necessary, and extract the fatty acids with *ether* in the Lindt apparatus for 3 hours, weigh the fatty acids, and deduct from the weight of the charge the weight of the fat and fatty acids. Remove the ether from the hydrolyzate by heating on the water bath.

Oxidation. Use for the oxidation as much *dichromate* as would be required for the complete combustion of the protein, and the proportion of dichromate to *sulfuric acid* used in the Beckmann oxidizing mixture, that is, about 10 g. of dichromate and 6 g. of sulfuric acid for each gram of protein. Conduct the oxidation in a Jena flask provided with a reflux condenser, heating on a hot plate for 5 to 6 hours. If the protein is rich in phenylalanine, white crystals form in the condenser which disappear toward the end of the oxidation.

Benzoic Acid Extraction. After cooling, transfer the mixture to the Lindt apparatus and extract with *ether* for 3 hours. Dry the ether over *fused sodium sulfate,* filter into a flat dish, and evaporate the ether at first on the water bath and finally at room temperature. The residue consists of a pungent liquid and a crystalline mass of benzoic acid together with isovalerianic acid and acetic acid. To the residue add *water saturated with benzoic acid* and allow to stand overnight. Collect the benzoic acid on a glass or porcelain Gooch crucible, wash once with *water saturated with benzoic acid,* and dry first in the air, then to constant weight at 60°. Recrystallization from anhydrous ether or chloroform does not add to the accuracy of the method.

Examples. Casein 2.68 to 3.4 (2.75 to 3.2 in the literature), fibrin 1.96 to 2.26 (1.2 to 2.5 in the literature), edestin 3.21 to 3.73 (2.4 to 4.0 in the literature), hemoglobin 3.4 to 3.74 (3.5 to 4.2 in the literature), zein 6.3 to 6.74 (4.87 to 6.96 in the literature), legumin 4.8 to 5.3 (none quoted from the literature), and gelatin 0.2 to 0.29% (0.4% in the literature) of phenylalanine.

Kapeller-Adler Hydroxylamine Hydrochloride Colorimetric Method.[131] As developed at the University of Vienna, the phenylalanine is nitrated to 3,4-dinitrobenzoic acid and converted by hydroxylamine in the presence of ammonia into a blue-violet ammonium salt of 3,4-di-isonitrodihydrobenzoic acid. Histidine is removed by precipitation with phosphotungstic acid and tyrosine is oxidized by potassium permanganate in the presence of sulfuric acid.

Apparatus. *Colorimeter.*

Reagents. *Nitrate Mixture.* Dissolve 10 g. of KNO_3 in 100 ml. of H_2SO_4.

Hydroxylamine Hydrochloride Solution, 15%.

Standard Phenylalanine Solution. Dissolve 0.1 g. of phenylalanine in 100 ml. of water rendered alkaline with NH_4OH; 1 ml. = 1 mg. of phenylalanine.

Process. *Hydrolysis.* Reflux 1.5 g. of the protein (3 g. of gelatin) for 20 hours with 7.5 ml. (15 ml. for gelatin) of *25% sulfuric acid*, filter the dark brown hydrolyzate, and dilute to 100 ml.

Histidine Precipitation with Phosphotungstic Acid. In order to precipitate the histidine and clear the solution by adsorption of the color on the precipitate, add *10% sulfuric acid* and *10% phosphotungstic acid* in the following amounts respectively: casein 18 and 45 ml., legumin 13.5 and 33.5 ml., edestin 22.4 and 56 ml., hemoglobin 15 and 37.5 ml., elastin 4.4 and 11.0 ml., and gelatin 15 and 37.5 ml. These amounts are so regulated as to avoid an excess of the phosphotungstic acid, which is detrimental.

After the addition of the reagents, dilute the solution to about 180 ml. and heat on a boiling water bath until the precipitated bases are nearly dissolved, then allow them to reprecipitate by cooling overnight. On the next morning, filter and wash with *water containing sulfuric acid,* then make up the filtrate to 200 ml. in a volumetric flask.

Tyrosine Oxidation with Potassium Permanganate. To an aliquot (usually 20 ml.) of the clear filtrate in a test tube or colorimeter tube with a 25-ml. graduation mark, add the cold *0.1 N potassium permanganate solution* to a faint rose color and evaporate in a porcelain dish on a boiling water bath to an oily consistency, taking care that the rose color persists.

Nitration. On cooling, add 2 ml. of *nitrate mixture* and heat on the boiling water bath for 20 minutes. Return the nitrated liquid to the 25-ml. test tube previously used, rinsing with a small amount of water to a total of about 6 to 9 ml.

Color Formation. Add with cooling in ice water 5 ml. of *15% hydroxylamine hydrochloride solution* and shake vigorously. Carefully make up to the mark with *ammonium hydroxide*, then cautiously mix, thus distributing through the liquid the violet coloration which appeared at the junction of the two liquids in the presence of alanine with evolution of gas. Warm the tube for 5 minutes in a beaker of water at 40°, at which temperature the color is less pronounced, then cool in ice water for 15 minutes to develop the maximum color.

Color Comparison. Compare the color with that of 1 to 4 ml. of the *standard phenylalanine solution* treated as in the actual analysis. A solution containing 0.1 mg. of phenylalanine in 15 ml. gives a distinct rose coloration.

Dihydroxyphenylalanine and Tyrosine

Arnow Nitrite-Molybdate Colorimetric Method.[132] The author (University of Min-

nesota) devised methods for the determination of 3,4-dihydroxyphenylalanine and tyrosine in the presence of each other.

APPARATUS. *Duboscq Colorimeter.*

REAGENTS. *Nitrite-Molybdate Reagent.* Dissolve 10 g. of $NaNO_2$ and 10 g. of $Na_2MoO_4 \cdot 2H_2O$ in 100 ml. of water.

Standard Phenylalanine Solution. Dissolve 50 mg. of 3,4-dihydroxyphenylalanine in 500 ml. in water. Add 2 ml. of 0.1 *N* HCl and water to the 1-liter mark. Preserve under toluene.

Alternative Standard. If 3,4-dihydroxyphenylalanine is not available, catechol may be used. Dissolve 192 mg. of catechol in water and make up to 1 liter. Preserve under toluene. Dilute 10 ml. of this stock solution to 100 ml. to make the standard. A green Wratten filter No. 61 (Eastman Kodak Co.) must be used in making readings with this standard; 1 ml. = 1 ml. of 3,4-dihydroxyphenylalanine standard described above.

PROCESS. *Color Formation.* In one test tube graduated at 5 ml., place 1 ml. of the unknown, containing 0.02 to 1 mg. of 3,4-dihydroxyphenylalanine, and in a second tube 1 ml. of the *standard solution.* Add to each in the order given, mixing after each addition: 1 ml. of *0.5 N hydrochloric acid,* 1 ml. of *nitrite-molybdate reagent* (a yellow color appears at this point), 1 ml. of *1.0 N sodium hydroxide solution* (red color), and water to 5-ml. mark.

Color Comparison. Compare the colors in the colorimeter.

HISTIDINE AND TYROSINE

Hanke Diazo Colorimetric Method.[133] Histidine is separated from tyrosine by precipitation as the silver salt and is determined by a method depending on the color formed with *p*-diazobenzene sulfonate. Tyrosine is determined in the filtrate by a colorimetric method in which hydroxylamine hydrochloride takes part (Chicago University).

APPARATUS. *Colorimeter.*

REAGENTS. *Diazo Reagent.* Prepare a solution (*A*) of 4.5 g. of sulfanilic acid in 45 ml. of HCl diluted to 500 ml. and a solution (*B*) of 25 g. of 90% $NaNO_2$ in water diluted to 500 ml. As needed, mix 1.5 ml. each of *A* and *B* in a 50-ml. volumetric flask, cool in ice for 5 minutes, then add 6 ml. of *B* and again cool 5 minutes, dilute to the mark, and keep on ice. Allow to stand at least 15 minutes, but not longer than 1 day. Store in Pyrex.

Color Standard. Add separately to 250 ml. of water 1 ml. of 0.5% Congo red solution and 1.1 ml. of 0.1% methyl orange solution, then dilute to 500 ml. This color mixture is for matching the color formed with histidine and histamine. It is also used in the Clifford method for determination of carnosine.

PROCESS. *Hydrolysis.* Mix 1 to 3 g. of the vacuum-dried protein with 100 ml. of water and 15 ml. of *95% sulfuric acid,* and reflux in a 300-ml. flask for 24 hours. Cool the mixture and transfer to a 3000-ml. flask. Dilute to 1500 ml., heat on boiling water bath, and treat the solution with a hot solution of 89 g. of *barium hydroxide* in 500 ml. of water. Adjust with *dilute barium hydroxide solution* and *dilute sulfuric acid* until it contains a slight excess of the sulfate ion. Digest for several hours and filter while hot through a Büchner funnel. Wash with hot water and transfer the filtrate to a glass dish, then concentrate on the steam bath.

Silver Precipitation. Transfer the semisolid residue to a 1-liter flask with 50 ml. of water, add 600 ml. of an *0.8% silver sulfate solution,* and mix thoroughly, then treat with a warm solution of 30 g. of *barium hydroxide* in 100 ml. of water. Place the brown mixture immediately in an ice bath where it is allowed to settle 30 minutes. Centrifuge the cold brown mixture (containing no noticeable deposit of metallic silver), decant the clear colorless supernatant liquid into a 2-liter flask, and immediately acidify the cold liquid with sufficient *20% sulfuric acid* to

cause the precipitate to settle. The liquid still contains a small amount of silver ion which produces a silver mirror with loss of tyrosine on warming. Oxidation does not occur in the acidified liquid. Wash the residue once by centrifuging with 50 ml. of cold *saturated barium hydroxide solution*, adding the washings to the main silver filtrate.

A. Histidine by the Koessler and Hanke Method.[134] *Treatment of Silver-Histidine Precipitate.* Transfer the precipitate to a 500-ml. flask with 60 ml. of *1.0 N sulfuric acid* and 150 ml. of water. Add 3 ml. of *hydrochloric acid*, agitate until homogeneous, and warm on the steam bath for 15 to 30 minutes, thus completing the conversion to silver chloride as indicated by the homogeneity and light color of the precipitate. Filter through a pleated paper into a 500- or 1000-ml. volumetric flask, wash with water until chloride-free, neutralize the filtrate with *20% sodium hydroxide solution*, and dilute to volume.

Color Formation. Pipet into one of the cylinders of the Duboscq colorimeter a volume of water (say 10 ml.) such that double that volume plus 7 ml. is a suitable quantity for the color comparison, then add from a pipet 5 ml. of *1.1% sodium carbonate solution* and 2 ml. of the *diazo reagent*. Mix by inclining the cylinder back and forth. Exactly 1 minute from the addition of the sodium carbonate add a volume of the unknown equal to that of the water previously added and mix as before.

Color Comparison. After 5 to 10 minutes, when the maximum is reached, match the color of the unknown set at 20 mm. with that of the *color standard* in the Duboscq instrument or its equivalent. The maximum color persists for only 1 to 10 minutes. Allow for the blank determination by deducting 0.3 ml. from the reading.

CALCULATION. Use: 1 ml. of the standard solution = 0.0370 mg. of histidine dichloride or 0.0133 mg. of histamine. Histamine may be separated by a method devised by the same authors.[135]

NOTE. The following solutions interfere with the reaction: 3% glycerol, 5% dextrose, 5% ethanol, 10% methanol, 10% methyl acetate, over 1% ammonium sulfate, and saturated aqueous solution of amyl alcohol.

B. Tyrosine by the Hanke and Koessler Method. *Treatment of Silver Filtrate.* Add to the solution, which has been acidified with *sulfuric acid, N hydrochloric acid* dropwise until a precipitate of silver chloride no longer forms and the liquid gives a faint test for chloride ion. Heat on the steam bath, adjust with *barium hydroxide* and *sulfuric acid* until a slight excess of sulfate ion is present, digest for several hours, filter, wash, and concentrate the filtrate and washings in a glass dish on the steam bath.

Mercuric Acetate Precipitation. Transfer the semi-solid residue, which usually contains tyrosine crystals, to a 300-ml. flask with 75 ml. of water, add 1 ml. of *glacial acetic acid* and 3.5 g. of *mercuric acetate*, and either reflux for 10 minutes or heat in the steam bath for 1 hour, thus forming a small precipitate. Cool, treat with 7.5 g. of *sodium chloride*, and cool in the refrigerator 2 hours. Transfer to a centrifuge tube with 20 ml. of *10% sodium chloride solution* and centrifuge for 5 minutes at high speed. Decant and discard the clear supernatant liquid. Wash the solid matter once with 25 ml. of *10% sodium chloride solution*, then transfer it back into the boiling flask with 25 to 50 ml. of hot *20% hydrochloric acid* and heat on the steam bath for 30 minutes, thus causing most and often all the solid matter to dissolve. Treat with 50 ml. of water and saturate with *hydrogen sulfide*. Heat on the water bath for 30 minutes and filter through a small pleated paper. Collect the filtrate and washings in a glass dish, evaporate on the steam bath to dryness, dissolve the pale brown crystalline residue in water, and dilute to 500 ml.

Hanke refers the reader to the thirty-five

page article by Hanke and Koessler [136] describing a microchemical colorimetric method for estimating tyrosine, tyramine, and other phenols.

TYROSINE TRYPTOPHAN

Nasse Millon Reagent Colorimetric Method for Tyrosine.[137] The original Nasse method employs as the reagent a *10% mercuric nitrate solution* and a few drops of *5% sodium nitrite*. After addition of the reagents to the unknown, the mixture is boiled. See Millon Mercuric Nitrate-Nitrite Test, Part I, C4*a*.

I. Weiss Modification for Tyrosine.[138] The details were worked out at Vienna-Gleichenberg.

REAGENTS. *Mercuric Sulfate Reagent.* A 10% solution of $HgSO_4$ in 5% H_2SO_4.

Standard Tyrosine Solution, 0.1% in 0.5% NaOH solution.

PROCESS. Pipet 3 ml. of the solution or hydrolyzate of the unknown into a test tube, add 2 ml. of *mercuric sulfate reagent* and 3 drops of *0.5% sodium nitrite solution,* then heat cautiously to incipient boiling and allow to cool. Within 5 minutes the characteristic brick-red coloration of the Millon test appears. Carry along with the test on the unknown one on 3 ml. of a 0.002% (1 + 50,000) tyrosine solution prepared by diluting 1 ml. of 0.1% solution to 50 ml.

Color Comparison. Compare the two solutions within 30 minutes after performing the test. No colorimeter is required. If the unknown shows a stronger reaction than the known, as is probable, dilute the original solution with an estimated volume of water and proceed as before with the diluted unknown and the undiluted known and continue to dilute and test until the solutions match.

CALCULATION. The procedure is best explained by an example. If the unknown measures 30 ml. and it is diluted first to 15 volumes, and that solution is diluted to 5 volumes, then the weight of tyrosine (T) in the 30 ml. of the solution is obtained by the following formula:

$$T = \frac{30 \times 15 \times 5}{50{,}000} \quad 0.045 \text{ g.}$$

If 30 ml. represents 1 g. of the protein, then the per cent of tyrosine is 4.50.

EXAMPLES. Casein 4.5, egg albumin 1.1% of tyrosine.

II. Fürth and Fischer Modification for Tyrosine.[139] Although Fürth,[140] after a study at Vienna University of the diazo, bromine addition, phosphomolybdic acid, and Millon reaction methods, reported unfavorably on all, later in cooperation with Fischer he devised the following procedure in which the Millon reaction is the chief feature. Earlier Fürth and Fleischmann [141] endorsed the bromine addition method.

APPARATUS. *Duboscq Colorimeter.*

REAGENTS. *Quinine Sulfate Reagent,* 5% solution in 5% H_2SO_4.

Millon Reagent.[142] Dissolve 1 part of metallic mercury in 2 parts of fuming nitric acid (sp.gr. 1.42) and dilute with twice its volume of water.

Standard Tyrosine Solution, 0.1% in 5% H_2SO_4.

PROCESS. *Hydrolysis.* Reflux 2.5 g. of the dry protein 12 hours with 3.5 ml. of *sulfuric acid* and 22.5 ml. of water.

Phosphotungstic Acid Precipitation. Dilute the hydrolyzate to 50 ml. in a volumetric flask and pipet 20 ml. (= 1 g. of the protein) into a graduated cylinder. Add *20% phosphotungstic acid solution* (20 to 40 ml.) until a precipitate no longer forms and allow to stand 1 day for the precipitate to settle. Accurately record the volume and filter through a dry small pleated paper into a graduated cylinder, then again note the volume.

If less than 30 ml. of phosphotungstic acid is required, make up to the mark in a 50-ml. volumetric flask, filter through a dry paper, and remove 40 ml. of the filtrate.

Quinine Precipitation. To the phosphotungstic filtrate, add *quinine sulfate reagent* until a precipitate no longer forms, avoiding a considerable excess, measure the volume, and filter through a dry pleated paper into a graduated cylinder. Again note the volume, add *30% sodium hydroxide solution* sufficient to precipitate the quinine, measure the volume, and filter through a dry paper. Note the volume, add *50% sulfuric acid* until acid to *litmus paper*, and note the final volume. Needless to say, careful measurements and a strict record of the several volumes are essential for accuracy.

Color Formation. To 10 ml. of the filtrate and 10 ml. of a *standard solution* of approximately the same tyrosine content, add 2 ml. of *Millon reagent* and allow to stand at room temperature 45 minutes. If several dilutions of the standard solution, ranging from 0.03 to 0.10% of tyrosine, are treated like the unknown, the comparison can be made with the one that comes nearest to matching.

Color Comparison. Filter, if necessary, and match the colors in a Duboscq colorimeter. It is essential that both the known and unknown be clear.

III. Lugg Modification for Tyrosine and Tryptophan.[143] By adding mercury salt to the acid solution of the hydrolyzate, Lugg (University of Adelaide) overcomes the interfering cloudiness formed in plant leaf protein hydrolyzates, when the details of the Folin and Ciocalteu modification are followed. His main procedure, however, is a modification of the mercury-nitrite (Millon) method. In a later paper,[144] he recommends hydrolysis with *5 N sodium hydroxide* at 110° for 20 to 24 hours with free access to air in the determination of tyrosine in the presence of diiodotyrosine and in the determination of tyrosine and tryptophan if much cystine is present.

REAGENTS. *Mercuric Reagent A.* Dissolve 75 g. of $HgSO_4$, 55 g. of $HgCl_2$, and 70 g. of $Na_2SO_4 \cdot 10H_2O$ in a mixture of 850 ml. of water and 125 g. of H_2SO_4, then dilute to 1 liter.

Mercuric Reagent B. Dilute 600 ml. of the strong solution (*A*) with an equal volume of 1.0 *N* H_2SO_4.

Mercuric Reagent C. Dissolve 12 g. of $HgSO_4$ and 9 g. of $HgCl_2$ in 600 ml. of water plus 100 ml. of H_2SO_4, then add with cooling 500 g. of H_2SO_4 and dilute to 1 liter.

Sodium Nitrite Solution, M. Dissolve 6.9 g. of $NaNO_2$ in water and dilute to 100 ml.

Standard Tyrosine or Tryptophan Solution. Dissolve 25 to 100 mg. of tyrosine in 0.1 *N* H_2SO_4 or 0.05 *N* NaOH and make up to 100 ml. with the same solvent. The solution keeps several months. Dissolve 25 to 100 mg. of tryptophan in water and make up to 100 ml. with water. The solution loses 1% in a week at 20°.

PROCESS. *Separation from Suspended Solids.* Centrifuge turbid hydrolyzates 10 minutes in a field of 1500 times gravity. Provide a glass rod 2 mm. in diameter and slightly bent at the end to stir solutions and suspend precipitates in them. Moisten rod slightly with *octyl alcohol* to use as a whisk to force solids at the air-liquid interface beneath the surface before centrifuging.

Place in a 15-ml. conical-bottom centrifuge tube the aliquot ranging up to 3 ml. of test solution, together with sufficient *5 N sulfuric acid* to bring the solution up to *p*H 0.3, as determined in a separate aliquot, using an indicator such as *cresyl blue.* Dilute to 5 ml. with *1.0 N sulfuric acid* or with appropriate volumes of *5 N sulfuric acid* and water. Add 5 ml. of *mercuric reagent A* and heat at 60 to 65° in a water bath for 30 minutes. Cool in a water bath at 1 or 2° below room temperature for 1 hour, centrifuge, and drain the clear liquid into a 25-ml. graduated cylinder. Run 10 ml. of *mercuric reagent B* into the centrifuge tube, stir up well for a minute or so, and again centrifuge. Drain into the cylinder and dilute with *mercuric*

reagent B to 24.5 ml. for the tyrosine determination. Reserve the precipitate in the centrifuge tube for the tryptophan determination.

Standard Solution. Prepare the standard simultaneously with the unknown and in the same manner.

TYROSINE. Within an hour, pipet 0.5 ml. of *M sodium nitrite solution* into the graduated cylinder so that it floats on the surface. As soon as possible shake both cylinders.

Color Comparison. Make the comparison of the unknown and the standard 3 minutes after mixing.

TRYPTOPHAN. Rub up the percipitate in the centrifuge tube (it may be left moist in the tube for a day without loss) with 10 ml. of *mercuric solution C* and heat at 40 to 45° in a water bath for 15 minutes with occasional rubbing of any solid matter that deposits. Cool in the water bath for 30 minutes at 1 to 2° below room temperature, centrifuge, and drain the clear solution into a 25-ml. graduated cylinder. Run another 10 ml. of *mercuric reagent C* into the tube, stir, and rub up any solid matter for a few minutes, then after centrifuging drain into the cylinder and dilute to 24.5 ml. with *reagent C*.

Standard Solution. Prepare a standard simultaneously, treating in the same manner.

Color Formation and Comparison. Within an hour or so, run 0.5 ml. of *M sodium nitrite solution* into each cylinder so as to float, shake, and make the color comparison with the least delay, the peak being reached within about 10 seconds.

Each milliliter of standard tyrosine and tryptophan solutions requires about 0.25 ml. of *5 N sulfuric acid* to bring it to pH 0.3. Prepare the color standards of tyrosine and tryptophan, if possible, from mixtures within, respectively, 70 and 150% of the intensities of the unknowns.

Introduce corrections for colored impurities present before the addition of the nitrite as determined in blanks in which water is substituted for nitrite.

CALCULATION. As in the original method.

IV. Zuwerkalow Modification for Tyrosine.[145] This author (Charkow) prefers mercuric sulfate to the nitrate.

APPARATUS. *Duboscq Colorimeter.*

REAGENT. *Hopkins Reagent.* A 10% solution of mercuric sulfate in 5% H_2SO_4.

PROCESS. To 1 ml. of a 1% solution of casein, egg albumin, or a globulin in *5% sodium hydroxide solution*, add 3 ml. of *glacial acetic acid*, then 2 ml. of *Hopkins reagent* and 1 drop of *0.5% sodium nitrite solution*. Heat over a free flame just to boiling.

Compare the color after 15 to 20 minutes in the Duboscq colorimeter with that of 1 ml. of *0.04% tyrosine solution* treated in like manner.

EXAMPLES. Casein 6.9, edestin 4.9, and egg albumin 4.4% of tyrosine.

V. Arnow Modification for Tyrosine.[146] This procedure (University of Minnesota), which permits the determination of tyrosine in the presence of 3,4-dihydroxyphenylalanine, depends on a modification of Millon reagent.

APPARATUS. *Duboscq Colorimeter.*

REAGENTS. *Mercuric Sulfate Reagent.* Dissolve 15 g. of $HgSO_4$ in 100 ml. of 5 *N* H_2SO_4.

Standard Tyrosine Solution. Dissolve 100 mg. of tyrosine in water and make up to 1 liter. Preserve under toluene.

PROCESS. *Color Formation.* In one test tube graduated at 5 ml., place 1 ml. of the unknown, containing 0.05 to 0.15 mg. of tyrosine, and in a second tube 1 ml. of the *standard tyrosine solution*. Add to each tube 1 ml. of *mercuric sulfate reagent*, mix, and immerse both tubes in boiling water for 10 minutes. Cool, add 1 ml. of *0.2% sodium nitrite solution* and water to the 5-ml. mark.

If 3,4-dihydroxyphenylalanine is present, the solution will be turbid, in which case centrifuge until clear.

If not centrifuged, let the solution stand for 5 to 10 minutes after the addition of the nitrite before comparing the colors.

Color Comparison. Pipet 3 to 4 ml. of the clear red supernatant liquid and compare with the standard tyrosine solution in the colorimeter. The Wratten filter aids in reading.

Folin and Denis Phosphotungstic-Phosphomolybdic Colorimetric Method for Tyrosine.[147] Numerous results in the literature, including those by Johns and Jones,[148] were obtained by this method developed at the Harvard Medical School. Nevertheless Gortner and Holm,[149] Thomas,[150] and others have shown that the color reaction is not specific and the results are often too high.

APPARATUS. *Duboscq Colorimeter.*

REAGENTS. *Folin and Denis Phenol Reagent.*[151] Reflux 20 g. of $20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$, 100 g. of $Na_2WO_4 \cdot 2H_2O$, and 50 ml. of 85% H_3PO_4 with 750 ml. of water for 2 hours, cool, and dilute to 1 liter.

Standard Tyrosine Solution, 0.02% in 0.1 N HCl; 5 ml. = 1 mg. of tyrosine.

PROCESS. *Hydrolysis.* Reflux 1 g. of the protein with 25 ml. of *20% hydrochloric acid* in a 500-ml. flask for 12 hours. Cool, transfer the hydrolyzate to a 100-ml. volumetric flask, make up to the mark, mix, and pipet 1 or 2 ml. into another 100-ml. volumetric flask.

Color Formation. To the aliquot add 5 ml. of *Folin and Denis phenol reagent* and, after 5 minutes, 25 ml. of *saturated sodium carbonate solution,* then make up to the mark with cold tap water.

Standard Solution. Treat 5 ml. of the *standard tyrosine solution* (containing 1 mg. of tyrosine) with 5 ml. of the *phenol reagent,* then add 25 ml. of *saturated sodium carbonate solution* and make up to the mark.

Color Comparison. After 10 minutes compare the colors of the unknown and standard in a Duboscq colorimeter, setting the known at 20 mm.

EXAMPLES. Gelatin trace, casein 6.5, ovovitellin (hen's egg) 5.2, ovomucoid (hen's egg) 5.4, conalbumin (hen's egg) 4.9, ovalbumin (hen's egg) 5.0, and lactalbumin (cow's milk) 4.9% of tyrosine.

I. Folin and Ciocalteu Macro Modification for Tyrosine and Tryptophan.[152] APPARATUS. *Colorimeter.*

REAGENTS. *Mercuric Sulfate Reagents, 15% in 6 N* H_2SO_4, and 1.5% in 2 N H_2SO_4. The salt must be absolutely pure.

Phenol Reagent. To 100 g. of $Na_2WO_4 \cdot 2H_2O$, 25 g. of $Na_2MoO_4 \cdot 2H_2O$, and 700 ml. of water in a 1500-ml. Florence flask, add 50 ml. of 85% H_3PO_4 and 100 ml. of HCl. Connect with a reflux condenser by a cork covered with tinfoil and boil gently 10 hours. Add 150 g. of $Li_2SO_4 \cdot H_2O$, 50 ml. of water, and a few drops of liquid bromine. Boil the mixture (without the condenser) about 15 minutes to remove the excess of bromine. Cool, dilute to 1 liter, and filter. Store the reagent (which should have no greenish color) protected from dust.

Standard Tyrosine Solution, 0.1% in 2 N H_2SO_4, containing 1 mg. of tyrosine per milliliter.

PROCESS. *Hydrolysis.* Weigh 1 g. of the protein into a 250-ml. Kjeldahl flask, add 2 ml. of *butanol* to prevent foaming, 2 silver wire spirals to prevent bumping, and 5 ml. of *20% sodium hydroxide solution.* Reflux in a water bath 18 to 20 hours with the cork covered by tinfoil. Add 10 ml. of water and boil 10 minutes to remove the alcohol. Remove the flame and immediately add from a pipet, dropwise but rather fast, 10 ml. of *14 N sulfuric acid,* thus precipitating the silicic acid and avoiding its colloidal solution. Shake well, cool, add 5 ml. more of *14 N acid,* transfer to a 100-ml. volumetric flask, rinse, and fill to the mark. Shake thoroughly and filter, keeping the funnel covered with a watch-glass. Keep the hydrolyzate in the refrigerator.

TYROSINE. *Precipitation of Tryptophan*

with Mercuric Sulfate. To 8 ml. of the hydrolyzate in a 15-ml. centrifuge tube, add dropwise from a height of about 3 cm. 4 ml. of *15% mercuric sulfate reagent.* After 2 to 3 hours, centrifuge for 5 minutes and decant the supernatant liquid into a 100-ml. volumetric flask, rinsing the edge of the tube with about 2 ml. of *0.1 N sulfuric acid.* To the precipitate, add 10 ml. of *1.5% mercuric sulfate reagent*, stir, and allow to stand 10 minutes. Rinse the rod with 2 ml. of *1.5% mercuric sulfate reagent*, centrifuge again, and transfer this wash liquid to the flask containing the original mother liquid, rinsing the edge of the tube.

Standard Solution. In a second 100-ml. volumetric flask, place 5 ml. of the *standard tyrosine solution*, add 4 ml. of *15% mercuric sulfate reagent*, 12 ml. of *1.5% mercuric sulfate reagent*, and about 7 ml. of *0.1 N sulfuric acid.*

Color Formation. To both standard and unknown add 6 ml. of *7 N sulfuric acid*, heat both flasks in a boiling water bath 15 minutes, cool under the tap to room temperature, and add with shaking 1 ml. of *2% sodium nitrite solution*, then dilute at once to the mark.

Color Comparison. Make the comparison without undue delay, always reading first the standard against itself to adjust the colorimeter.

Calculation. If the standard is set at 20 mm. and the hydrolyzate contains 1% of protein, obtain the percentage of tyrosine (P) by the formula

$$P = \frac{20 \times 1.25 \times 5}{R}$$

in which R is the reading.

Tryptophan. Follow one of the two procedures for the treatment of the mercury precipitate above.

Procedure A. To the tryptophan mercury precipitate in the centrifuge tube add 10 ml. of *N hydrochloric acid* and stir with a slender glass rod 3 mm. or less in diameter. Heat in a beaker of boiling water 10 minutes, pass into the hot solution *hydrogen sulfide*, in a slow current through a glass tube with a drawn-out end, until a black precipitate of sulfide is obtained, rinsing the rod and tube with 2 ml. of *10 N sulfuric acid.* Stopper the centrifuge tube and let stand 1 hour.

Centrifuge, decant into a 100-ml. volumetric Pyrex flask, and rinse the edge of the tube with 2 ml. of *0.1 N sulfuric acid.* Add 10 ml. of *0.1 N sulfuric acid* to the hydrogen sulfide residue, stir, rinse the rod with 2 ml. of *0.1 N acid*, and centrifuge again. Add this wash liquid to the mother liquid in the 100-ml. flask, then add about 15 ml. of water, boil rapidly 4 to 5 minutes to remove the hydrogen sulfide, and cool.

Standard Solution. In a second 100-ml. volumetric flask, place 5 ml. of the *standard tyrosine solution*, containing 1 mg. of tyrosine per milliliter, and dilute with 35 ml. of water.

Color Formation. To both standard and unknown, add 25 ml. of *saturated sodium carbonate solution*, dilute the unknown to 65 to 70 ml., and add to each flask with shaking 5 ml. of *phenol reagent.* After allowing to stand 30 minutes, fill to the mark and mix.

Color Comparison. Set the standard at 20 mm. and compare with the unknown, always first reading the standard against itself.

Calculation. Obtain the milligrams of tryptophan (P) in 1 g. of the protein from the formula

$$\frac{20 \times 12.5}{R \times 0.887}$$

in which R is the reading of the unknown.

Procedure B. Stir the washed tryptophan mercury precipitate with 10 ml. of *0.1 N sulfuric acid* and wash the rod with 2 ml. of *0.1 N acid.* Throw down the precipitate again by centrifuging and remove the wash liquid by decantation. Add this second wash liquid to the flask containing tyrosine and add a similar amount of *0.1 N sulfuric acid* to the standard.

As in the first procedure, add to the precipitate 10 ml. of *1.0 N hydrochloric acid*, stir, heat in a boiling water bath 30 minutes, cool, and rinse into a 100-ml. volumetric flask with 30 ml. of water, then add 25 ml. of *saturated sodium carbonate solution.*

Standard Solution. Prepare a standard as in the first procedure.

Color Formation. Add 5 ml. of *phenol reagent* to each flask (unknown and standard) and allow to stand 30 minutes, then add to each 2 or 3 ml. of *5% sodium cyanide solution* (to intensify the color) and dilute to volume.

Color Comparison. Make the comparison as under Procedure A.

CALCULATION. Instead of dividing the tyrosine equivalent to the tryptophan by the theoretical factor 0.887, reduce the divisor by 5% to 0.843. This is necessary because the liberated mercury salt diminishes the depth of color.

EXAMPLES. The following results were obtained on tyrosine and tryptophan respectively: casein 6.37 and 1.4, egg albumin 4 and 1.3, edestin 4.53 and 1.51, gliadin 3.1 and 0.84, and zein 5.9 and 0.17%.

NOTE. Raoul[153] recommends a preliminary extraction with hot *90% ethanol* for 4 hours to remove amino-phenols such as hordenine and tyramine, also pigments, followed by an *ether* extraction for 3 hours. Hydrolysis is performed by heating with *20% sodium hydroxide* in a glycerol bath for 20 hours, using *butanol* to regulate the boiling. Storing the neutralized hydrolyzate for 48 hours at 5° in the refrigerator is regarded as essential. The color reading is made 6 minutes after adding the nitrite, when the maximum intensity is reached, and not after 22 minutes when it begins to decline.

II. Folin and Marenzi Micro Modification for Tyrosine and Tryptophan.[154] APPARATUS. *Colorimeter.*

REAGENTS. See Folin and Ciocalteu Modification above.

PROCESS. *Hydrolysis.* Place in a 150 x 16 mm. Pyrex test tube 100 mg. of dried protein, add 2 ml. of *20% sodium hydroxide solution*, and shake gently until dissolved. Close with a cork wrapped in tinfoil, carrying a 50-cm. tube to act as a condenser, and heat in a boiling water bath for 12 to 14 hours (albumins) or 16 to 18 hours (globulins). Add to the hot solution 3 ml. of *7 N sulfuric acid*, thus obtaining the silica as a precipitate and not as a colloidal solution. Cool, transfer to a 25-ml. volumetric flask and dilute to the mark. Add 0.2 to 0.5 g. of *kaolin*, shake well, and filter through a 9-cm. paper, keeping the funnel covered with a watch-glass in the intervals.

Tryptophan Precipitation with Mercuric Sulfate. To 20 ml. of the hydrolyzate in a conical 50-ml. centrifuge tube, add 4 ml. of *15% mercuric sulfate reagent in 6 N sulfuric acid* dropwise from a height of at least 3 cm. and allow to stand 2 to 3 hours for precipitation of the tryptophan. Centrifuge for 5 to 10 minutes, thus causing the precipitated tryptophan with a little tyrosine and nearly all the cystine derivative to deposit, leaving the tyrosine in the liquid. Reserve the precipitate for the determination of tryptophan below.

TYROSINE. Decant the solution into a 100-ml. volumetric flask, washing the edge of the tube with 1 ml. of *0.1 N sulfuric acid.* Add 10 ml. of *1.5% mercuric sulfate reagent in 2 N sulfuric acid* to the precipitate, stir with a glass rod, and rinse the rod with 2 ml. of the *reagent.* After 10 minutes, centrifuge as before, rinse the edge of the tube with 1 ml. of *0.1 N sulfuric acid,* and add the washings to the main solution. Repeat the treatment, using, however, 10 ml. of *0.1 N acid,* then 2 ml. additional for rinsing the rod.

Standard Solution. To another 100-ml. volumetric flask, add 4 ml. of the *standard tyrosine solution in 2 N sulfuric acid,* 16 ml. of water, 4 ml. of *1.5% mercuric sulfate reagent,* 12 ml. of *1.5% mercuric sulfate reagent,* and 14 ml. of *0.1 N sulfuric acid.*

Color Formation. Finally add to the unknown and standard 6 ml. of *7 N sulfuric acid* so that the entire contents is equivalent to about 100 ml. of N sulfuric acid. Heat both flasks for 5 minutes in a boiling water bath, cool, add with shaking 1 ml. of *2% sodium nitrite solution*, after 2 minutes dilute to the mark, and shake.

Color Comparison. Compare known and unknown in a colorimeter.

CALCULATION. Obtain the milligrams of tyrosine (M) present in the 20-ml. hydrolyzate by the following formula:

$$M = \frac{4 \times 20}{R}$$

in which R is the colorimetric reading with the standard set at 20 mm.

TRYPTOPHAN. To the precipitate in the centrifuge tube add 10 ml. of *N hydrochloric acid* and heat in a water bath for 30 minutes. Cool the solution, together with possibly a trace of insoluble matter of unknown composition, filter through a small paper into a 100-ml. volumetric flask, and wash up to about 60 ml.

Standard Solution. Add to another 100-ml. volumetric flask 1 mg. of tryptophan and dilute to 60 ml.

Color Formation. Add to each flask 25 ml. of *saturated sodium carbonate solution* which has not been in contact with rubber, mix, add 5 ml. of *phenol reagent*, mix again, and after 30 minutes add 2 or 3 ml. of *5% sodium cyanide solution* to intensify the color. Dilute to the mark and mix.

Color Comparison. Compare known, set at 20, and unknown in a colorimeter.

CALCULATION. Obtain the milligrams of tryptophan (M) in the 20 ml. of hydrolyzate by the following formula:

$$M = \frac{1 \times 20}{R \times 0.843}$$

in which R is the colorimeter reading in millimeters. The factor 0.843 is explained in the foregoing Folin and Ciocalteu modification.

Millar Method for Tyrosine.[155] **I. Plimmer and Eaves Modification.**[156] In the original Millar method, the titration is carried out with 0.2 M sodium bromate, which is too strong for obtaining exact readings when the tyrosine content is low (0.01 to 0.04 g.). Plimmer and Eaves, after investigations at University College, London, and the University of Sheffield, rectified this defect without reducing the amount of reacting bromine by using a solution ten times weaker (0.02 N = 0.033 M) and conducting the operation in a closed flask. Now that micro burets are available, the reduction in strength of the standard solution is not so imperative for accuracy.

REAGENT. *Standard Sodium Bromate Solution,* 0.02 N (= 0.0333 M). Dissolve 5.033 g. of $NaBrO_3$ in water and dilute to 1 liter.

PROCESS. To the hydrolyzate containing in a closed flask tyrosine dissolved in *hydrochloric acid*, add 10 to 15 ml. of *20% potassium bromide* and allow to stand 10 to 15 minutes in a closed flask.

Titrate with *0.02 N sodium bromate solution* to a persistent yellow color. If the solution before titration was colored so as to obscure the end-point, use *starch solution* and *potassium iodide* as indicators at this stage. Back-titrate with *0.02 N thiosulfate solution.*

CALCULATION. Theoretically 1.765 g. of bromine, corresponding to 0.5558 g. of sodium bromate, are required by 1 g. of tyrosine. Two-thirds of a mole of sodium bromate is required to react with one mole of tyrosine, hence 1 ml. of 0.03334 M sodium bromate is equivalent to $(181.09/1000) \times 0.03334 \times \frac{2}{3} = 0.00424$ g. of tyrosine.

II. Bonicatti Modification.[157] The procedure is briefly as follows: Add to the solution containing the tyrosine *hydrochloric acid* to acid reaction, 5 ml. of *20% sodium bromide solution,* and *0.1 N sodium bromate solution* until the solution is yellow. After 2

hours, add an excess of *potassium iodide* and titrate the liberated iodine with *thiosulfate solution*. Tryptophan and phenylglycine interfere.

Folin and Looney Phosphotungstic–Molybdic Colorimetric Method for Tyrosine and Tryptophan.[158] Apparatus. *Colorimeter.*

Reagents. *Hopkins and Cole Reagent:* 10% $HgSO_4$ in 5% H_2SO_4.

Phenol Reagent. Boil a solution of 15 g. of MoO_3 and 10 g. of NaOH in 200 ml. of water until it no longer smells of ammonia. Add 100 g. of $Na_2WO_4 \cdot 2H_2O$, 50 ml. of 85% H_3PO_4, 100 ml. of HCl, and water to make 800 ml. Reflux 10 hours, add a few drops of bromine to decolorize, and boil without the condenser to remove the excess of bromine, then cool, filter, and dilute to 1 liter.

Standard Tyrosine Solution, in 5% H_2SO_4; 1 ml. = 1 mg.

Standard Tryptophan Solution, in 5% H_2SO_4; 1 ml. = 1 mg.

Process. *Hydrolysis.* Introduce into a 300-ml. long-neck Kjeldahl flask 1 g. of the purified protein, previously dried for 48 hours in a vacuum desiccator over sulfuric acid. Add 3.5 g. of *crystalline barium hydroxide* and 25 ml. of water, close the neck with a Hopkins condenser, and reflux the mixture over a micro-burner for 40 to 48 hours. Add 30 ml. of *20% sulfuric acid* and heat the flask in boiling water 30 to 60 minutes to drive off any hydrogen sulfide that may have formed. Cool, transfer to a 100-ml. volumetric flask, dilute to the mark, mix, and filter through a dry paper into a dry

Separation of Tyrosine and Tryptophan. Transfer an aliquot of 1 to 10 ml. of the hydrolyzate (representing 0.01 to 0.10 g. of the protein) to a centrifuge tube graduated at 10 ml., add 2 ml. of *Hopkins and Cole reagent*, and dilute to the mark with *5% sulfuric acid*. Insert a rubber stopper and shake a few times. After standing 2 hours, centrifuge and decant the supernatant liquid containing the tyrosine from the sediment which contains the tryptophan.

A. Tyrosine. *Color Formation.* Transfer 5 ml. of the liquid (half of the aliquot) to one 100-ml. volumetric flask and add 1 ml. of *standard sulfuric acid-tyrosine solution* (containing 1 mg. of tyrosine) to a second 100-ml. volumetric flask. To the second flask add also 1 ml. of *Hopkins and Cole reagent* and 3 ml. of *5% sulfuric acid.* To each flask add about 30 ml. of water, 20 ml. of *saturated sodium carbonate solution*, and, with shaking, 4 ml. of *5% sodium cyanide solution*, then 2 ml. of the *phenol reagent* and mix.

Color Comparison. After allowing to stand 30 minutes, make the color comparison in the usual manner with the standard set at 20 mm.

Calculation. Obtain the milligrams of tyrosine (M) in the aliquot of the hydrolyzate by the following formula:

$$M = \frac{20 \times 2}{R}$$

in which R is the reading of the unknown.

B. Tryptophan. The tryptophan precipitate dissolves in an excess of sodium cyanide only under certain conditions and the color obtained is deeper than the color for the same weight of tryptophan not previously treated with mercury, hence it is necessary to treat the tryptophan standard like the unknown, precipitating it with *mercury sulfate* at the same time or before the precipitation is made with the unknown. As several days have no effect on the standards, they may be made and kept in well-stoppered centrifuge tubes.

Color Formation. To the unknown mercury tryptophan precipitate and to a similarly prepared and centrifuged standard, containing 1 mg. of tryptophan, add 10 ml. of water, stopper, and shake so as to form a suspension. Within 2 or 3 minutes, add 4 ml. of *5% sodium cyanide solution* to both

standard and unknown, stopper, and mix, the solution being immediate if the preliminary shaking is not omitted. Transfer the standard and unknown to 100-ml. volumetric flasks, rinsing to a volume of about 50 ml. Add 20 ml. of *sodium carbonate solution* and then, with shaking, 2 ml. of *phenol reagent.*

Color Comparison. After allowing to stand 30 minutes, dilute at once to volume and make the comparison, with the standard set at 20 mm.

CALCULATION. Obtain the milligrams of tryptophan (M) in the aliquot of the hydrolyzate by the following formula:

$$M \quad 20$$

in which R is the reading in millimeters of the unknown.

Tillmans, Hirsch, and Stoppel Xanthoproteic Colorimetric Method for Tyrosine and Tryptophan.[159] The yellow color produced by the action of nitric acid on certain proteins is due to the presence of one or more of the amino acids having the benzene ring, namely tryptophan, tyrosine, and phenylalanine, the sensitivity to the test being in the order named. Certain prolamines (sturin, clupein, salmin, etc.) contain no amino acids with the benzene ring and consequently do not respond to the test.

In the method devised by Tillmans *et al.* at the University of Frankfurt, a/M, the conditions are regulated to prevent the interference of phenylalanine and give full play to the action of tryptophan and tyrosine. Hydrolysis is omitted, since the heating with 0.1 N nitric acid for 2 hours accomplishes the same purpose. The manipulation is simple, although involving colorimetric readings at low and high hydrogen ion concentrations, and the calculation is not difficult when the somewhat unusual details are mastered.

APPARATUS. *Colorimeter.*

REAGENTS. *Standard m-Nitrophenol Solution.* Dissolve 50 mg. in 500 ml. of 0.1 N NaOH. For practical purposes, the color value of this solution is equivalent to that of an alkaline standard solution containing 2.5 mg. of tyrosine per 500 ml. after nitration.

Sørensen Buffer Solution.

PROCESS. *Nitration.* Weigh into an Erlenmeyer flask 1 g. of the protein, or a quantity of protein matter or amino acid mixture containing tyrosine and tryptophan (say 25 mg.), add 400 ml. of *0.1 N nitric acid,* attach some form of condenser, and heat for 2 hours in a boiling water bath. Cool, neutralize to *litmus* with *sodium hydroxide,* transfer to a 500-ml. volumetric flask, and fill to the mark.

Color Comparison at pH 1.8. Remove to a colorimeter tube by a pipet an aliquot of 20 ml. of the solution, add 20 ml. of water and 10 ml. of *0.1 N hydrochloric acid.* This mixture has a pH of 1.8, as shown on the accompanying table. Compare with 50 ml. of the *standard m-nitrophenol solution* diluted to one-tenth strength containing 0.5 mg. of the dye, and record the color strength in terms of milligrams of the dye as S'.

Color Comparison at pH 11.5. Remove to another colorimetric tube 5 ml. of the solution, add 1.65 ml. of *0.1 N sodium hydroxide solution* (see table), and compare with 38 ml. of the *standard solution* diluted to one-quarter strength containing 0.95 mg. of *m-nitrophenol.* Record the color strength in terms of milligrams of the dye as S''.

CALCULATION. Let a' and b' represent the color values of tyrosine and a'' and b'' the color values of tryptophan at pH 1.8 and 11.5 respectively; also let x and y represent the milligrams of tyrosine and tryptophan in the unknown; then

$$a'x + b'y = S'$$

and

$$a''x + b'' = S''$$

Since a', b', a'', and b'' may be derived from the table, it only remains to solve the equation for x and y.

pH	Color Value *		pH	Color Value *	
	Tyrosine	Tryptophan		Tyrosine	Tryptophan
1.8	0.10	0.40	7.0	2.5	0.85
2.1	0.20	0.41	8.4	4.2	0.95
2.5	0.35	0.43	9.5	6.2	1.10
3.2	0.50	0.50	10.0	6.5	1.20
4.0	0.63	0.60	11.5	7.0	1.23
5.0	0.88	0.64	12.9	7.4	1.30
6.0	1.50	0.70			

* Color intensity compared with that of 1 mg. of tyrosine.

NOTE. Provided the gap is sufficiently wide, the solutions may be adjusted to other pH values than 1.8 and 11.5 in accordance with the following directions and the corresponding color values derived from the table.

For pH 1.8, 2.1, and 2.5, add *hydrochloric acid* to 0.02, 0.01, and 0.002 *N* respectively. For pH between 3.2 and 10.0, add *Sørensen Buffer solution.* For pH 11.5, 11.7, and 11.9, add respectively 3.3, 5.3, and 8.5 ml. of *0.1 N sodium hydroxide solution* per 100 ml. total volume and for pH 12.7 and 12.9, add respectively 5.3 and 8.5 ml. of *0.1 N sodium hydroxide solution* per 10 ml. total volume.

TYROSINE AND TYRAMINE

Gerngross, Voss, and Herfeld Nitroso-Naphthol Colorimetric Method.[160] The original method is based on the deep purple color formed when 1 ml. of a solution of 1 g. of tyrosine, tyramine, or other substituted para-phenols, 6 ml. of a *10% solution of anhydrous sodium carbonate* in 1 liter, and 1 drop of *1% α-nitroso-β-naphthol in ethanol* are heated to boiling, and 1 or 2 drops of *nitric acid* are added. Since the color fades rapidly and does not obey Beer's law, the determination is made on successive dilutions of the unknown until the color is only faintly perceptible, then compared with the color obtained on treating a solution containing 1 γ of tyrosine in 10 ml. in like manner.

Maciag and Schoental Iron Ammonium Alum Modification.[161] By adding a saturated solution of the alum to the colored solution, Maciag and Schoental stabilize the color so that the reading can be made after 1 hour, both for tyrosine and tyramine.

APPARATUS. *Pulfrich Photometer,* with 1-cm. cell and filter S 50.

REAGENT. *α-Nitroso-β-Naphthol Solution,* 1% in ethanol.

PROCESS. *Color Formation.* To 2 ml. of an aqueous solution of the tyrosine or tyramine, a bleached blood filtrate, or presumably a protein hydrolyzate, add 1 drop of *1% ethanolic α-nitroso-β-naphthol solution* and heat to boiling, then add 3 drops of *nitric acid* and heat again to boiling. As soon as the red-violet color is formed, add to the mixture

Tyrosine or Tyramine γ/ml.	Extinction	
	Tyrosine	Tyramine
1.25	.....	0.137
2.5	0.076	0.149
5.0	0.125	0.208
7.5	0.161	0.256
10.0	0.208	0.328
12.5	0.244	0.357
15.0	0.292	0.432
17.5	0.328	0.488
20.0	0.377	0.538
22.5	0.444	0.620
25.0	0.509	0.658
37.5	0.678	0.939
50.0	0.824	1.187
0	.....	0.076 *

* No alum, 0.086.

2 ml. of *saturated ferric ammonium sulfate solution* and heat again to boiling.

Color Reading. After 1 hour, read the extinction coefficient in the Pulfrich photometer, using a 1-cm. cell and filter S 50. Find the corresponding concentration of the tyrosine and tyramine in the table above.

Lautenschläger Titanic Acid Volumetric Method.[162] The method (Heidelberg University) is applicable for the determination of histidine and other imidazole derivatives.

Reagent. *Standard Titanium Trichloride Solution,* 0.1 *N*. Standardize solution against standard 0.1 *N* $FeNH_4(SO_4)_2 \cdot 12H_2O$.

Process. *Hydrolysis.* Two methods were found to give good results: (1) hydrolysis with *hydrogen iodide* and *red phosphorus* followed by separation by *silver acetate* and (2) acid hydrolysis with separation by *mercuric chloride.* Only the second and simpler method is given.

Reflux a weighed portion of the protein by heating in an oil bath with 10 parts by weight of *hydrochloric acid* or *33% sulfuric acid* to which at the start a small amount of *stannous chloride* has been added to remove humus substances as formed. Add to the hydrolyzate sufficient *concentrated mercuric chloride solution* to supply at least 2 molecules for 1 molecule of the histidine thought to be present, then add *sodium carbonate* to alkaline reaction, filter, and wash. Suspend the precipitate in water, precipitate the mercury with *hydrogen sulfide,* filter, wash, and remove the excess of hydrogen sulfide by evaporation.

Diazobenzene-Sulfonic Acid Treatment. To an aliquot of 10 ml., containing about 0.1 g. of histidine reduced to small volume, add 20 ml. of *ethanol* and an excess (usually 10 ml.) of freshly prepared *2% diazobenzene-sulfonic acid solution* to which *sodium hydroxide* has been added to alkaline reaction. Evaporate the colored alkaline solution on the water bath until the ethanol is completely removed and acidify with *dilute hydrochloric acid.*

Titanium Trichloride Titration. The procedure is essentially that devised by Knecht and Hibbert[163] for similar purposes.

To the colored acid solution add an excess of *standard 0.1 N titanium trichloride solution* (usually 20 ml.), heat for a short time, then cool and titrate the excess of the titanic acid with *0.1 N ferric ammonium sulfate solution,* using *ammonium sulfocyanate* as indicator and conducting the titration in an inert gas as described by Knecht and Hibbert.

Blank. Conduct a blank determination, using 10 ml. of *2% diazobenzene-sulfonic acid,* 20 ml. of *ethanol,* and 20 ml. of *0.1 N titanium trichloride solution,* and titrating back with *0.1 N ferric ammonium sulfate solution.*

Calculation. One molecule of the dye formed from the tyrosine and imidazole derivatives requires one molecule of titanic trichloride for its reduction. Introduce the correction found in the blank determination.

Kapeller-Adler Bromine-Ammonia Colorimetric Method.[164] The method (Vienna University) depends on the blue-violet color formed when a solution of histidine is treated with an acetic acid solution of bromine followed by ammonia and ammonium carbonate.

Apparatus. *Pulfrich Photometer,* with 5-mm. cell and filter S 50.

Reagents. *Bromine Reagent.* Dissolve 5 g. of bromine in 500 ml. of glacial acetic acid and dilute with 1500 ml. of water.

Ammonium Carbonate Reagent. Mix 2 volumes of NH_4OH and 1 volume of 10% ammonium carbonate solution.

Standard Histidine Solution. Dissolve 100 mg. of histidine in 2 ml. of 10% H_2SO_4 and dilute to 100 ml.; 1 ml. = 1 mg. of histidine.

Process. *Hydrolysis.* Since the method was applied by Kapeller-Adler directly to

urine and other biological fluids, no method of hydrolysis was formulated.

Permanganate Decolorization. Into a tube with a 15-ml. graduation mark, pipet a portion of the solution representing a definite weight of the sample. Add *0.1 N potassium permanganate solution* dropwise until a faint rose color persists. If in a short time the rose color fades and a yellow color persists, add dropwise more *permanganate;* if, however, the solution is colorless, omit the second addition. In any event, the solution must be clear and colorless. If a manganese deposit forms, heat the tube for a few minutes in a beaker of water until it goes into solution.

Bromine Treatment. To the clear colorless cold solution add the *bromine reagent* dropwise until a pale yellow color forms, adding more of the *reagent* if, after a short time, the color fades.

Color Formation with Ammoniacal Reagent. When the yellow color has persisted for 10 minutes, add to the solution 2 ml. of the *ammonium carbonate reagent* and heat the tube in a beaker of boiling water for 5 minutes. Cool the intensely violet solution, thereby causing the maximum development of color. Fill to the mark and mix.

Color Measurement. After 30 minutes from the time the ammoniacal reagent was added, measure the color transmission in the 5-mm. cell of the Pulfrich photometer, using filter S 50, and compare with a curve plotted for 0 to 3.75 mg. of histidine per 15 ml, in 0.25 increments as abscissas and 0 to 1.0 negative log of the transmission ($1/T$) as ordinates from data obtained by the method used for the unknown.

Block Nitranilic Acid Method.[165] Block, after carrying out some eighty-odd experiments at the New York State Psychiatric Institute and Hospital, adopted the following procedure.

PROCESS. *Hydrolysis.* Hydrolyze 5 g. of material such as gelatin, casein, or cattle blood, with 50 ml. of *8 N sulfuric acid.* Dilute the hydrolyzate to 100 ml. in a volumetric flask.

Precipitation with Silver Sulfate. Pipet an aliquot of 50 ml. of the hydrolyzate into a centrifuge tube, dilute to 200 ml., add warm *concentrated barium hydroxide solution* to pH 3.5 to 4, remove the barium sulfate by centrifuging, and wash twice with hot water. Concentrate the clear filtrates to about 100 ml. *in vacuo,* precipitate the histidine with *silver sulfate* at pH 7.4, and centrifuge. Decant the solution and wash the histidine silver with water.

Removal of Silver. Suspend the silver salt in water, acidify with *dilute sulfuric acid,* precipitate the silver with *hydrogen sulfide,* filter, and wash.

Nitranilic Acid Precipitation. Concentrate the filtrate containing histidine sulfate solution to about 200 ml., adjust the pH of the solution to 3.5 to 4 with *dilute barium hydroxide solution,* filter, and wash the precipitate. Concentrate the filtrates to 10 ml. and precipitate the histidine from a 50% methanol solution by an excess of *solid nitranilic acid.* Let stand overnight, filter the histidine nitranilate on a sintered-glass crucible, wash with *methanol* and *ether,* dry at 110°, and weigh.

Potassium Chloride Precipitation. Treat a 100-mg. portion of the histidine nitranilate (except that from gelatin) obtained above with a mixture of 90 ml. of water, 85 ml. of *acetone,* 1 ml. of *1.0 N hydrochloric acid,* 1 ml. of *5 N sulfuric acid,* and 10 ml. of *25% potassium chloride solution.* Filter and wash the precipitate of dipotassium nitranilate with *2.5% potassium chloride solution in 50% ethanol.* Determine nitrogen in the filtrate.

CALCULATION. Obtain the weight of histidine from the weight of histidine nitranilate by the factor 0.403; also from the weight of nitrogen in the filtrate from the precipitated dipotassium nitranilate.

Proline

Engeland Chloroaurate Gravimetric Method.[166] The treatment of the hydrolyzate is in four stages: (1) methylation to betaine stachydrine, (2) separation of stachydrine by mercuric chloride in hydrochloric acid solution, (3) formation of stachydrine chloroplatinate, and (4) conversion into stachydrine chloroaurate, which is weighed.

Process. *Hydrolysis.* Conduct the hydrolysis with *hydrochloric acid* in the usual manner, remove the acid by evaporation on the water bath, and take up the dry residue in *methanol* and *10% ethanolic potassium hydroxide solution.*

Methylation. Employ for the methylation *methyl iodide* in preference to methyl sulfate, thus avoiding the formation of resins on subsequent precipitation with mercuric chloride.

Stachydrine Chlorohydrate Formation. Treat the methylated product with *moist silver chloride*, thus replacing the iodine by chlorine and obtaining stachydrine chlorohydrate (dimethylproline chlorohydrate). Remove the silver iodide by filtration and evaporate the filtrate to dryness.

Mercuric Chloride Precipitation. Take up the stachydrine chlorohydrate in the smallest volume of water necessary for solution, add *hydrochloric acid* to a distinct acid reaction, and precipitate with *saturated mercuric chloride solution.* Collect and wash the dense white crystalline precipitate on a filter. To remove the mercury compounds other than the stachydrine mercury substance, dissolve the precipitate in *hydrochloric acid*, remove the mercury by precipitation with *hydrogen sulfide*, filter, and wash the precipitate. Evaporate the filtrate to dryness and take up the residue in *absolute ethanol*, decanting from any insoluble matter that may be present.

Platinic Chloride Precipitation. Treat the ethanolic solution with *ethanolic platinic chloride solution* and store in the refrigerator for several hours to cause a complete precipitation of the yellow chloroplatinate. Decant the supernatant liquor onto a filter, wash with *absolute ethanol* two or three times by decantation, and finally remove the ethanol by heating on the water bath.

Since the platinum compound is too deliquesent for accurate weighing, convert it into chloroaurate as follows: Dissolve the precipitate in a few milliliters of water, precipitate the platinum with *hydrogen sulfide*, filter, wash, and evaporate the filtrate to dryness.

Auric Chloride Precipitation. Take up in a few drops of *hydrochloric acid* and add *30% auric chloride solution.* Store in the refrigerator for 5 or 6 hours to precipitate completely the stachydrine chloroaurate. Filter rapidly and wash the crystals with an ice cold saturated solution of stachydrine aurate, dry, and weigh.

Correct the weight for the solubility of mercury stachydrine salt in mercuric chloride solution (60 γ/ml.) and for the solubility of stachydrine chloroaurate in hydrochloric acid (13 mg. per ml. at 18 to 20°).

Calculation. Correcting for an obvious typographical error, the formula assigned to stachydrine chloroaurate by Engeland and Bastian differs from that of proline in that $(CH_3)_2$ and $AuCl_4$ instead of H are attached to N. This formula corresponds to a gold content of 40.83%. They do not state what precautions must be taken in drying the gold salt nor do they give a factor for conversion to proline which, according to the writers' calculation, is 0.2382. The determination of the gold in the salt and the adoption of a conventional factor would obviate the questionable assumption of a valence of 5 for Au as well as N.

Van Slyke Indirect Method.[167] Although one of the earliest, the method still holds its own in point of accuracy. As here outlined it applies especially to casein.

Process. *Hydrolysis and Esterification.* Hydrolyze 500 g. of the protein, esterify by the Fischer method, and distil three times

with *barium hydroxide solution* by the Levene and Van Slyke method.[168] Extract with *absolute ethanol* the amino acids yielded by the esters (about 400 g.) boiling below 90° at 0.5 mm. pressure and filter. Concentrate on the water bath as far as possible, then take up in cold *absolute ethanol*, filter, and repeat the process. Evaporate to remove the ethanol and take up in water.

Total and Amino Nitrogen Determination. Make up to a definite volume, remove aliquots, and determine the total nitrogen by the Kjeldahl method and the amino nitrogen by the Van Slyke method.

CALCULATION. Subtract the percentage of amino nitrogen from the percentage of total nitrogen and multiply the difference by the appropriate factor.

Proline is the only amino acid in the ethanol fractions that does not react with nitrous acid.

EXAMPLE. Proline in casein 6.70%.

PROLINE AND SERINE

Lang Hypochlorite Colorimetric Method.[169] Sodium hypochlorite converts proline and oxyproline respectively into pyrroline and hydroxypyrroline. On steam distillation, the two products are determined together in the distillate by measuring the red color formed with dimethylamino-benzaldehyde and the hydroxypyrroline is determined by measuring the violet color formed with isatin. Pyrroline, and therefrom proline, are obtained by difference. Serine gives the same color reaction as proline, hence the result by the Lang method includes the two amino acids, necessitating a deduction of the serine content if the proline content alone is desired.

APPARATUS. *Steam Distillation Apparatus.* A cylindrical reaction chamber (5), provided with a stopcock (6) at the bottom and closed by a triple-bored stopper at the top, is connected on one side with a coil (1) for superheating steam and on the other with an upright condenser. The third hole of the stopper carries a tube extending to the bottom of the chamber into which a funnel tube (3) with a stopcock (4) delivers. A three-way stopcock (2) is in the tube joining 1 and 5.

Pulfrich Photometer, with filter S 57.

REAGENT. *Raschig Hypochlorite Reagent.*[170] Weigh accurately a 1-liter flask containing 190 ml. of Kjeldahl NaOH solution and 600 g. of ice. Run a stream of chlorine into the mixture until the increase in weight reaches 71 g. Filter the mixture and dilute the filtrate to 1 liter. The solution keeps one month.

PROCESS. *Hydrolysis.* Reflux for 20 hours about 300 mg. of the protein with 5 ml. of *6 N hydrochloric acid.* After cooling, add a little purified *kaolin* to the liquid and make up to 10 ml. in a volumetric flask. Remove 1 to 5 ml. (according to the proline content) of the hydrolyzate to a test tube, neutralize accurately to *phenolphthalein*, with *6 N sodium hydroxide solution*, and cool in ice water.

Hypochlorite Treatment. Into the reaction chamber (5) of the steam distillation apparatus with stopcocks (4 and 6) closed, run superheated steam (120 to 130°) with the three-way stopcock (2) so adjusted that only the communication between 1 and 5 is open. As soon as the apparatus is heated, add to the aliquot of the neutralized hydrolyzate in the test tube 0.1 ml. of the *Raschig hypochlorite reagent* for each 10 mg. of proline represented and pour into the reaction chamber through the funnel tube (3) provided with a reservoir for ice water. Open stopcock 4 and at the same time adjust the three-way stopcock so that there is communication between the reaction flask and the outside air, thus avoiding excess pressure in the former, then adjust stopcocks 2 and 4 as before. Rinse the test tube twice with 2 to 3 ml. of water and pour through the funnel tube into the reaction flask.

The beginning of the decomposition of the

amino acids is indicated by a pronounced foaming. Collect in a graduated cylinder exactly 100 ml. of distillate containing the volatile products of the reaction. At the end of the operation, empty the chamber through stopcock 6 and rinse the chamber for the next determination.

Pipet an aliquot (preferably 20 ml.) of the distillate into a conical centrifuge bottle and add dropwise 2 ml. of *saturated mercuric chloride solution*. When the precipitate begins to settle, whirl and decant off the supernatant liquid. Wash the precipitate with two 5-ml. portions of *ethanol*, each time stirring the precipitate, heating at 60° on the water bath, cooling, centrifuging, and decanting. Finally mix the precipitate with about 2 ml. of water, dissolve with the addition of a few drops of *5% sodium cyanide solution*, add 1 ml. of *6 N hydrochloric acid*, and wash into a 25-ml. (if the amount of proline is small, 10-ml.) flask.

To one aliquot, add 1 ml. of *2.5% ethanolic p-dimethylamino benzaldehyde solution*, to the other, 1 ml. of *0.05% isatin solution in sulfuric acid*. Fill both flasks to the mark with water and suspend in a boiling water bath. After exactly 5 minutes, remove the flask with the aldehyde reagent from the bath and cool in ice water, but allow the flask with the isatin reagent to remain there 5 minutes longer.

If the protein contains proline, the aldehyde reagent produces a red-violet coloration; if, however, oxyproline is also present, the isatin reagent also produces a coloration.

Color Reading. After cooling, measure the color value of both solutions in the Pulfrich photometer, using filter S 57.

CALCULATION. Neither color solution obeys Beer's law strictly. Obtain data for a calculation graph, treating as described proline- and oxyproline-free proteins to which are added progressive amounts (0.5 to 3.0 mg. in 0.5 mg. increments) of the two amino acids. Prepare three curves: one for the isatin reaction of oxyproline and one each for the aldehyde reaction of oxyproline and of proline.

In calculation of the results from the graph, first find in the curve for the isatin reaction the amount of oxyproline corresponding to the extinction coefficient, then find in the curve for the aldehyde reaction with oxyproline the amount of oxyproline corresponding to the extinction coefficient. By difference, obtain the extinction coefficient for the aldehyde reaction of proline, then find in the corresponding curve the amount of proline.

TRYPTOPHAN

Herzfeld Methylamino-Benzaldehyde Colorimetric Method.[171] Rhode[172] observed that tryptophan forms with *p*-dimethylamino-benzaldehyde a blue coloration, but Herzfeld (Zürich University) was the first to apply the reaction in an analytical method. Kurchin,[173] Thomas,[174] and May and Rose[175] modified the method but, as shown by Holm and Greenbank,[176] they, as well as Herzfeld, failed to secure the maximum color intensity in the standard solution.

APPARATUS. *Duboscq Colorimeter* or *Color Comparison Tubes.*

REAGENTS. *Aldehyde Reagent.* Dissolve 20 g. of *p*-dimethylamino-benzaldehyde in 500 ml. of HCl and add to 500 ml. of water.

Standard Color Solution. Dissolve 1 g. of anhydrous $CuSO_4$ in 100 ml. of water. Mix 1 ml. of the solution with 20 ml. of NH_4OH and dilute to 100 ml.

PROCESS. *Pancreatin Digestion.* To 1 g. of the protein, add 0.5 g. of *pancreatin* of known tryptophan content dissolved in 500 ml. of *0.5% sodium carbonate solution* and protect with suitable amounts of *chloroform* and *toluene*, forming layers below and above the liquid, then digest for 24 hours in an incubator. After digestion, filter through a dry paper into a dry flask.

Color Formation. Mix 50 ml. of the filtrate with 10 ml. of the *aldehyde reagent*, then

make up to 100 ml. in a volumetric flask with *hydrochloric acid.* Allow to stand 30 hours and filter through a double paper.

Color Comparison. Match the blue color against the *standard color solution.* The reaction is delicate to 0.1 mg. of tryptophan in 100 ml.

CALCULATION. The color of the standard solution is equivalent to that obtained with 0.1 mg. of tryptophan treated as described.

EXAMPLES. The results on various proteins, obtained by Herzfeld, have been shown by Holm and Greenbank to be much too low; the last-named authors, however, by decreasing the time of development found the method satisfactory, as shown below.

Holm and Greenbank Modification.[177] These authors (U. S. Dept. of Agriculture) modified slightly the original method.

REAGENTS. See above.

Standard Color Solutions, containing 2 and 5 mg. of tryptophan.

PROCESS. Suspend 0.10 g. of the finely divided protein in 100 ml. of *20% hydrochloric acid* containing an excess of the *aldehyde reagent.* Digest at 37° and read the color from time to time against standards containing 2 and 5 mg. of tryptophan, treated in a Duboscq colorimeter in like manner, until the color of the unknown reaches its maximum intensity.

EXAMPLES. Casein 2.24, fibrin from blood 5.00, and Witte's Peptone 5.40% of tryptophan by the Holm and Greenbank method. Other authors obtained respectively as follows: Folin and Looney 1.54, 2.90, and 3.03, Fürth *et al.* 2.02, 5.30, and 5.30, Herzfeld 0.51, 1.05, and 1.25%; on casein only, Hopkins and Cole 1.50, Thomas 1.7 to 1.8, and May and Rose 1.50%.

NOTE. Sullivan, Milone, and Everitt[178] accelerate the color formation by heating at 85° and the use of *dilute hydrogen peroxide.* In this way the maximum color formation is secured in 30 seconds instead of 7 to 8 days.

Kraus (Mrs. Ragins) Vanillin Colorimetric Method.[179] Miss Kraus (University of Chicago) proposed in her first paper a method based on the blue color formed by a solution of tryptophan when allowed to react with an acid solution of vanillin; in her later paper she gives slight changes. If applied to the mercuric sulfate precipitate, the standard, as well as the unknown, must be treated alike. Indole and skatole, if present, must be removed by extraction with toluene. Proline and proline-containing proteins in low concentrations do not interfere.

REAGENTS. *Acid Vanillin Reagent,* 0.5%. Dissolve 0.5 g. of vanillin in 100 ml. of 50% acetic acid.

Mercuric Sulfate Reagent, 2% solution in 5% H_2SO_4.

Pancreatin, U.S.P.

PROCESS. *Hydrolysis.* Weigh 0.5 g. of the protein and 0.25 g. of *pancreatin* into a small Erlenmeyer flask containing 25 ml. of *0.4% sodium carbonate solution,* add *toluene* as a preservative, and incubate for 5 days at 37°.

Color Formation. Transfer an aliquot of the hydrolyzate to a 15-ml. centrifuge tube, precipitate with *mercuric sulfate reagent,* centrifuge, and wash. To the washed precipitate in the tube add 1 ml. of *mercuric sulfate reagent* (to intensify the color) and 0.4 ml. of *acid vanillin reagent,* then gradually with mixing 12 ml. of *hydrochloric acid.* Stopper and allow to stand 24 hours together with a *standard* containing 0.2 mg. of tryptophan treated in like manner.

Color Comparison. Match the color of the unknown against that of the standard solution.

EXAMPLES. Gliadin 0.60, secalin 0.52, sativin 0.13, hordein 0.60, flaxseed protein 0.90, excelsin 1.27, edestin 1.27, and casein 1.23% of tryptophan.

Strepkov and Mavlianov Modification.[180] APPARATUS. *Micro Colorimeter.*

REAGENTS. *Standard Color Solution.* Mix 10 ml. of a solution containing 0.5 g. of

methyl red in 375 ml. of ethanol, 5 ml. of a solution containing 0.5 g. of methylene blue in 400 ml. of ethanol, and 10 ml. of 15% H_2SO_4, then dilute to 250 ml. For use, dilute 5 ml. to 100 ml. with ethanol.

PROCESS. *Color Formation.* Heat on a boiling water bath for 5 minutes 2 ml. of a solution containing 3.75 to 7.5 mg. of tryptophan in 100 ml. with 0.5 ml. of *2% ethanolic vanillin solution* and 2 ml. of *sulfuric acid.*

Color Comparison. Cool the solution and compare with the *standard color solution.*

CALCULATION. Multiply the height of the column of the standard solution by 0.0465 and divide by the height of the column of the unknown, thus determining the weight of tryptophan in the solution.

Shaw and McFarlane Glyoxylic Acid Colorimetric Method.[181] The method was worked out at Macdonald College.

APPARATUS. *Evelyn Photoelectric Colorimeter.*

REAGENTS. *Glyoxylic Acid Solution.*[182] To 100 ml. of 5% oxalic acid solution in a 150-ml. beaker add 3 ml. of $N/5$ $HgCl_2$ solution and a few small pieces of aluminum wire. Cover with a watch-glass and heat on a boiling water bath 5 minutes after the first appearance of bubbles on the wire. Let stand 5 minutes further at room temperature, filter, add 2 ml. of H_2SO_4 to the filtrate, and store in the refrigerator. The solution remains stable 2 weeks or longer.

Standard Tryptophan Solution. Prepare a standard solution (blank) containing tryptophan in equal volume and concentration to the test solution and treat in the same way as the unknown, except that the addition of glyoxylic acid is omitted.

CURVE. Prepare calibration curves corresponding to 540- and 520-μ wave lengths respectively, showing for each wave length as abscissas 0.02 to 0.14 mg. of tryptophan and as ordinates percentages of light transmission from 0 to 100. Except for the highest concentration, the solutions obey Beer's law.

PROCESS. *Solution.* As applied to casein, dissolve in *10 or 20% sodium hydroxide solution* or *5% acetic acid.* Hydrolyzed under pressure, tryptophan is unstable in sodium hydroxide but stable in barium hydroxide.

Color Formation. Place 0.10 to 2.00 ml. of the solution, containing 0.005 to 0.150 mg. of tryptophan, in a 10-ml. glass-stoppered measuring cylinder, and add 0.5 ml. of *glyoxylic acid solution* and 0.5 ml. of *M/25 copper sulfate solution.* Make up to 3 ml. with water and add from a buret 5 ml. of *sulfuric acid* in portions of 0.5, 1.0, 1.5, and 2.0 ml., shaking under the tap after each addition. Allow to stand for 10 minutes in cold tap water, then heat in boiling water for 5 minutes. Cool to room temperature and dilute to 10 ml. with *5 + 3 (by volume) sulfuric acid.*

Color Reading. Transfer to a clean dry colorimeter tube and take the readings after 15 minutes. Compare with the curve.

Roth and Schuster Xanthoproteic Colorimetric Method.[183] The method (I. G. Farbenindustrie) depends on the xanthoproteic reaction produced in a hot oxidizing mixture that destroys interfering substances.

APPARATUS. *Pulfrich Photometer,* with filter S 43 and 3-ml. cell.

REAGENTS. *Oxidizing Reagent.* Mix 100 ml. of 70% H_2SO_4 and 60 ml. of 25% HNO_3.

Standard Tryptophan Solution, 0.1%.

PROCESS. A. TOTAL TRYPTOPHAN. *Oxidation.* Weigh 10 g. of a pulped fresh fruit or vegetable or 1 g. of a ground cereal or dry legume, well mixed with 10 ml. of water, into a large test tube or a small Erlenmeyer flask. Add from a buret in a fine stream 40 ml. of the *oxidizing reagent* with shaking. Allow to stand 10 minutes at room temperature, then place in a boiling water bath where the mixture is shaken occasionally during 1 hour. Remove from the bath, allow to cool, filter through a Schleicher and Schüll No. 589 paper into a 50-ml. volumetric flask, and wash up to the mark with *70% sulfuric acid.*

Treat aliquots of a 0.1% tryptophan solu-

tion, representing 1 to 10 mg. of tryptophan, in the same manner as the unknown.

Color Measurement. Read the extinction value (E) in the Pulfrich photometer, using filter S 43 and 3-ml. cell.

CALCULATION. Compare the extinction of the unknown with a curve plotted with E as ordinates and milligrams of tryptophan from 1 to 10 as abscissas. The solution obeys Beer's law.

B. BOUND AND FREE TRYPTOPHAN. *Treatment with Trichloracetic Acid.* Weigh into a centrifuge tube the same amount of the sample as used for determining the total tryptophan, diluting if necessary to 10 ml. Add 50 ml. of *5% trichloracetic acid,* mix well, and centrifuge 30 minutes. Wash the deposit twice in the centrifuge tube with 50 ml. of *1% trichloracetic acid,* then remove it with 8 ml. of water to an Erlenmeyer flask and proceed with the bound tryptophan as directed above.

If in the decanted trichloracetic acid there is a relatively high content of free tryptophan (e.g., cauliflower with 0.019%), concentrate the solution to 10 ml. *in vacuo* and proceed as under Total Tryptophan; but if the content is low (e.g., spinach with 0.003%), start anew with 100 g. of the pulped vegetable in a 200-ml. flask, add *10% trichloracetic acid* to the mark, shake well, and centrifuge for 30 minutes. Remove 100 ml. of the supernatant liquid and evaporate to 10 ml. at 100° under diminished pressure.

CALCULATION. Determine the color extinction (E) of the bound tryptophan, then subtract from that of the total tryptophan to obtain the free tryptophan.

EXAMPLES. Total tryptophan: Cauliflower 0.045 (free 0.019), spinach 0.054 (free 0.003), soy bean seed 0.550 (free 0.032), carrot none, potato 0.046, grass 0.097, wheat 0.200, and corn (maize) 0.110%.

Shimada Bromine Colorimetric Method.[184] The method is based on the reaction of 4 atoms of bromine with 1 molecule of tryptophan and is claimed to be specific for free tryptophan.

APPARATUS. *Duboscq Colorimeter.*

REAGENTS. *Acid Bromine Solutions.* (A) Bromine 1.5% in 33% acetic acid. (B) Bromine 0.3% in 33% acetic acid.

PROCESS. *Trichloracetic Acid Extraction.* Mix a suitable portion of the tryptophan solution with an equal volume of *16% trichloracetic acid* and filter. Pipet 2 ml. of the mixture and 2 drops of *methyl orange* into a separatory funnel containing 4 ml. of *chloroform* and 1 ml. of *glacial acetic acid,* previously mixed by shaking.

Bromination. To the mixture add slowly from a buret with cautious shaking *acid bromine solution A,* thus causing the formation of a red coloration which passes into the chloroform layer, while the methyl orange in the aqueous layer is gradually decolorized. It is desirable that the color intensity of the chloroform layer be less than that of the aqueous layer, thus preventing the overoxidation of red-colored substances produced by the bromine solution.

When the color of the methyl orange in the aqueous layer is nearly discharged, add another drop of *indicator,* then dropwise *acid bromine solution B* until the red color of the dye disappears entirely.

Shake the mixture vigorously, draw off the chloroform layer into a 10-ml. graduated cylinder, wash in like manner with 1 ml. of *chloroform,* add the washing to the cylinder, and make up to the mark with *10% acetic acid in chloroform,* thus avoiding the formation of a turbidity. Shake well the contents of the cylinder and keep for 15 to 20 minutes in an incubator at 37°.

Color Comparison. Use for the comparison a *standard solution of tryptophan* treated as described. It is claimed that a tryptophan content in 1 ml. of 0.51 to 5.1 mg. (M/0.0025 to 0.025) can be determined with an error of about 3%.

Glutathione

When discovered by Hopkins,[185] glutathione, as the name suggests, was considered to be a dipeptide, but later [186] it was shown to be a tripeptide with amino acids combined as follows:

$$HOOC\cdot CHNH_2\cdot CH_2\cdot CH_2\cdot CO—$$
Glutamic acid

Cysteine

$$NH\cdot CH_2\cdot COOH$$
Glycocoll

The reduced and oxidized forms in which it exists in tissues are believed to play important roles in reduction and oxidation biological processes.

Methods of at least three types have been proposed: (1) *iodometric* with removal of proteins by sulfosalicylic acid alone,[187] or by trichloracetic acid in conjunction with zinc sulfate [188] or cadmium lactate,[189] (2) *colorimetric*, employing phosphotungstic acid,[190] and (3) *colorimetric* employing nitroprusside.[191]

Woodward and Fry Sulfosalicylic Acid Iodometric Method for Blood and Tissues Containing No Ascorbic Acid.[192] The method (University of Pennsylvania) was designed for blood which does not contain sufficient ascorbic acid to influence the result. The Woodward Manometric Method (below), since glyoxalase is employed to separate glutathione from ascorbic acid and other interfering substances, gives results on certain tissues 40 to 70% lower than by the Woodward and Fry method.

Reagents. *Potassium Oxalate Solution*, 30%. As little as 0.01 ml. oxalates 1 ml. of blood.

Sulfosalicylic Acid Solution, 1 *M* and 4%. Prepare a 1 *M* solution by weighing out 25 g. (because of deliquescence) instead of 22 g., diluting with water, and making up to 100 ml. Prepare a 4% solution by diluting 45.6 ml. of the 1 *M* solution to 250 ml.

Potassium Iodate Solution, 0.001 *N* in 2% sulfosalicylic acid. Prepare a 0.005 *N* stock solution (which keeps indefinitely) by dissolving 0.1783 g. of the salt in water and diluting to 1 liter. Mix, each week, 50 ml. of the stock solution and 22.8 ml. of 1 *M* sulfosalicylic acid solution and dilute to 250 ml.

Process. *Solution*. Oxalate 1 volume of blood and lake thoroughly with 8 volumes of water, add after 5 to 10 minutes 1 volume of *1 M (22%) sulfosalicylic acid solution* very slowly with shaking, and filter through a dry paper.

Iodate Titration of Reduced Glutathione, Direct. To 10 ml. of the filtrate add 2.5 ml. of *4% sulfosalicylic acid solution*, 2.5 ml. of *5% iodine-free potassium iodide solution*, and 2 drops of *1% soluble starch solution*, then titrate with *0.001 N potassium iodate solution containing 2% sulfosalicylic acid*. The reading represents the reduced glutathione. During the titration keep the flask in water at 19 to 20°.

Iodate Titration of Total Glutathione. Treat the 12 to 15 ml. remaining after the first 10 ml. aliquoting above with 30 to 40 mg. of *zinc dust*, allow to react 20 minutes, filter through a dry paper, and proceed with a 10-ml. aliquot as before, thus obtaining a reading for total glutathione, which, less the direct reading, represents the oxidized glutathione.

Examples. In normal blood, reduced glutathione 25 to 41 and oxidized glutathione 3 to 11 mg. per 100 ml.

Woodward Glyoxylase Manometric Method for Tissues Containing Ascorbic Acid.[193] Apparatus. *Barcroft-Warburg Manometric Assembly*. The cups have a single side bulb of 0.8 ml. capacity and a total volume of 18 ml. The manipulation is discussed by Platt and Schroeder.[194]

Reagents. *Acetone-Yeast*, glutathione-free. Prepare by washing the usual product by the Albert, Buchner, and Rapp procedure [195] or, for small amounts, as needed ac-

cording to the directions of Platt and Schroeder.[194] Large amounts are conveniently washed and dried by the acetone-ether process.

For use in the manometer estimation choose an amount which, with 0.1 mg. of glutathione, will give in 20 minutes carbon dioxide equivalent to about three-fourths of the capacity of the manometer. Usually 0.5 ml. of a 15 to 20% suspension furnishes the desired amount of the enzyme.

Methylglyoxal Solution. Prepare a solution of high concentration as directed by Bernhauer and Görlich.[196] Determine the methylglyoxal content by the *m*-nitrobenzoylosazone procedure of Spoehr and Strain [197] and dilute the solution to a content of 10 mg. per ml.

STANDARDS. Preliminary to the plotting of a curve, determine the effects of increasing amounts of pure glutathione on the activity of glutathione-free acetone-yeast glyoxalase. Weigh suitable amounts of glutathione standards as follows: 0.025, 0.05, 0.1, and 0.15 mg. Conduct a blank on the yeast with no glutathione; this takes care of any pressure developed because of the side reactions between methylglyoxalase and yeast as well as change in pressure due to temperature and barometric variations. After tipping, allow an open period of 4 minutes, then read every 5 minutes for 20 minutes. Plot the carbon dioxide produced in each standard (less the blank) against the time, thus obtaining a straight-line curve, indicating a uniform carbon dioxide production every 5 minutes.

If the line is straight only for the first 15 minutes, extrapolate the remainder. In a typical graph given by Woodward the abscissas are 0.025, 0.05, 0.10, and 0.15 and the ordinates 50 to 300 by 50 increments.

PROCESS. Place an amount of the unknown, containing not more than 0.1 mg. of glutathione, in the side bulb and measure the activity it produces with the same amount of *yeast* as in the standard and in the same manner. If only one actual analysis is made, this may be run with the standard, omitting the 0.15-mg. solution when the multiple apparatus has 6 manometers. If the actual analyses are run as a separate series, make a yeast blank along with these. When a sulfosalicylic acid protein-free filtrate is used for the analysis, neutralize this to *methyl orange* in the side tube with *0.2 M sodium bicarbonate solution* as determined by a separate titration.

Use the following amounts respectively of 2% sulfosalicylic acid blood and tissue filtrate dilutions: Whole blood 0.5 ml. and 1 + 5; red blood cells 0.5 ml. and 1 + 10; blood plasma 0.5 ml. and 1 + 1.5; animal tissue extract 0.25 and 1 + 5.

After measuring the volume of carbon dioxide produced in 20 minutes by the unknown, read the amount of glutathione present directly on the curve.

Since the factor here studied is glutathione, place this in the side bulb. In setting up a run, measure the materials first into the main chamber of each manometer cup as follows: 0.5 ml. of *glutathione-free acetone-yeast* (15 to 20% suspension), 0.2 ml. of *methylglyoxal* (10 mg. per ml.), 0.4 ml. of *sodium bicarbonate solution* (0.2 *M*), and water to a total of 2 ml. including measurements in the side bulb. Mix the yeast and methylglyoxal solution first, thus cutting down the observed blank gas evolution caused by a slight reaction between these two, since the greater part of the reaction occurs before the manometric readings can be started.

Langou and Marenzi Phosphotungstic Acid Colorimetric Method.[198] Cystine solution is used as the standard, since equimolecular solutions of ascorbic acid and of cystine and glutathione reduced with sodium sulfite produce with the Folin reagent the same color.

APPARATUS. *Colorimeter.*

REAGENTS. *Standard Cystine Solution.* Dissolve 40 mg. in 200 ml. of 0.2 *N* H_2SO_4.

Folin Phosphotungstic Acid Reagent. Re-

flux for 2 hours 10 g. of molybdate-free $Na_2WO_4 \cdot 2H_2O$, 80 ml. of 85% H_3PO_4, and 750 ml. of water, cool, and make up to 1 liter with water.

PROCESS. *Solution.* Treat blood directly or grind minced meat or organs with *10% trichloracetic acid solution*, filter, and make up to volume.

Color Formation. Prepare three tubes as follows: In tube 1 place 2 ml. of *standard cystine solution;* in tubes 2 and 3 place an aliquot of the trichloracetic acid filtrate containing 0.7 to 2.5 g. of reducing substances. Add to tube 3 *sodium carbonate solution* (20%) to alkaline reaction and allow to stand 1 hour, thus destroying the ascorbic acid. To all three tubes add 0.2 ml. of *20% sodium sulfite solution,* then after 2 minutes add 0.2 ml. of *20% lithium sulfate solution* followed by 2 ml. of *Folin reagent* and 2 ml. of *20% sodium carbonate solution.* After 4 minutes dilute all three tubes to 25 ml. with *2% sodium sulfite solution.*

Color Comparison. Compare tube 2, containing the total glutathione plus ascorbic acid, and tube 3, containing glutathione only, with the standard cystine solution in tube 1.

Fujita and Iwatake Meta-Phosphoric Acid Iodometric Method.[199] The authors (Kitasato Institute, Tokyo) prevent the liberation of iodine by iron in the iodometric titration by deproteinizing with meta-phosphoric acid and avoid the disturbing influence of postmortem-formed cysteine by freezing. If only the glutathione content is desired, they prefer the nitroprusside method because it eliminates the color due to ascorbic acid; but, by obtaining results by both methods and subtracting one from the other, a result for ascorbic acid is obtained which agrees closely with that for ascorbic acid by the 2,6-dichlorophenolindophenol titration method.

PROCESS. *Meta-Phosphoric Acid Treatment.* Grind thoroughly a suitable weight of the tissues (meat, etc.) with *sand,* 4 parts by weight of *5% meta-phosphoric acid solution* (prepared daily and kept in the refrigerator), and 5 parts by weight of water. Mix thoroughly, centrifuge, and filter through a dry paper. Dilute a measured volume of the filtrate to 10 times that volume with water.

Titration. Dilute a suitable number of milliliters of the solution (v) with *2% meta-phosphoric acid solution* to 20 ml. Add 1 ml. of *25% potassium iodide solution* and 1 ml. of *0.01 iodine solution,* and allow to stand 3 minutes. Titrate with *0.005 N sodium thiosulfate solution.* Conduct a blank determination in like manner.

CALCULATION. Obtain the milligrams of glutathione (G) in 100 g. of the sample by the following formula:

$$G = \frac{1535A - a}{v}$$

in which A and a are respectively the milliliters of thiosulfate required for the blank and actual determination, and v is the number of milliliters of the unknown used in the titration.

c. ACID AMIDES

(Glutamine and Asparagine)

See also Part II, C1 and H7.

Of the acid amides, or rather the amino acids characterized by the single terminal COOH and $CO(NH_2)$ groups, two members, namely glutamine or aminoglutamic acid $[CO(NH_2) \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH]$ and asparagine or aminosuccinamic acid $[CO(NH_2) \cdot CH_2 \cdot CH(NH_2) \cdot COOH]$, are here treated. They are widely distributed in the vegetable kingdom, especially in vegetables and fruits, but have not been isolated from proteins.

Vickery, Pucher, Clark, Chibnall, and Westall Amide Hydrolysis Colorimetric Method.[200] As regards glutamine, the method is a modification of the Chibnall and Westall

Method.[201] The amide group of glutamine is completely hydrolyzed in 2 hours at 100° within *p*H 6 to 7, whereas that of the asparagine is only slightly affected.

APPARATUS. *Pulfrich Spectro Photometer.*

Pucher, Vickery, and Leavenworth Ammonia Distillation Apparatus.[202]

REAGENTS. *Buffer Solution,* for *p*H 6 to 9.[203] Prepare a solution containing 0.1 *M* KH_2PO_4 and 0.05 *M* borax.

Borax Reagent. Dissolve 5 g. of borax in 100 ml. of 0.5 *N* NaOH.

Nessler Reagent (Modified Nessler-Folin Reagent).[204] Dissolve 22.5 g. of iodine in 20 ml. of water containing 30 g. of KI, add 30 g. of pure metallic mercury, and cool by shaking under the tap. When the supernatant liquid has lost the yellow color due to iodine, decant and make sure that it still contains free iodine, as shown by adding a few drops to 1 ml. of a 1% starch solution, adding dropwise more of the iodine solution if necessary. Dilute to 200 ml., mix, and add to 975 ml. of exactly 10% NaOH, mix again, and allow to stand until clear.

PROCESS. *Solution.* One of four methods may be employed for plant tissues, although each has its defects.

(1) Extraction with cold water after cytolysis of the fresh tissues with *ether*, as described by Chibnall,[205] employing the technique of Pucher, Vickery, and Leavenworth.[206] To obtain complete extraction, the residue from the hydraulic press must be thoroughly ground in a plate-type mill before washing. Coagulation of proteins at 80° is also probably desirable.

(2) Grinding with *sand*, heating at 80°, cooling, filtering, and washing. This method is suited for small samples of succulent tissues.

(3) Freezing with protection against loss of water, thawing, and washing. Suited for small amounts only.

(4) Drying at a controlled temperature in an oven.

A. GLUTAMINE. *Hydrolysis.* Pipet an aliquot of the solution, not exceeding 5 ml., into a 25 x 200 test tube together with 10 ml. of a *buffer solution* at *p*H 6.5. Close the tube with a rubber stopper carrying a 20-cm. length of 1-mm. bore heavy-wall glass tubing, moisten both stopper and orifice of the tube with a few drops of water, and heat in a boiling constant level water bath for exactly 2 hours.

Distillation. Cool, allow a few drops of water to suck through the capillary tube for rinsing, transfer to the flask of the Pucher *et al.* ammonia distillation apparatus, rinse with 20 ml. of water, add 3 ml. of *borax reagent*, and distil *in vacuo* at 40°.

Color Formation and Measurement. Dilute the distillate, treat with 5 ml. of *Nessler reagent*, make up to 50 ml. in a volumetric flask and determine the extinction coefficient in the Pulfrich spectrophotometer.

CALCULATION. Correct for a blank. Compare with a calculation curve plotted from data obtained with solutions of differing concentrations of glutamine or corresponding amounts of ammonium hydroxide.

B. ASPARAGINE. Mix an aliquot of 5 ml. of the solution, or a smaller aliquot diluted to 5 ml., with 1 ml. of *6 N sulfuric acid* and hydrolyze for 3 hours at 100° as described above. Transfer to the distillation apparatus with 20 ml. of water, add 5 ml. of *1.0 N sodium hydroxide solution*, followed by 5 ml. of *alkaline borax reagent*, distil the ammonia, and determine nitrogen with *Nessler reagent* as described above. Correct for the blank, thus obtaining the total amide nitrogen. To determine the asparagine nitrogen, deduct the sum of the free ammonia nitrogen and the glutamine nitrogen and apply the conventional factor for nitrogen to asparagine.

d. PURINES; BASES

See Part II, C1, H1, H3, and I1, I2, and I3.

e. Ammonia

Ammonia is one of the cleavage products liberated in the hydrolysis of proteins. It is also formed in various animal and vegetable foods during ripening or decomposition. The smell of ammonia is often quite distinct in certain types of cheese, such as Camembert, where the ripening has been carried to such a degree as to cause profound decomposition of the proteins. Ammonia is also formed to some extent in meat and eggs during storage and in excessive amounts during spoilage.

Ammonium carbonate and ammonia alum at one time were extensively used in baking powders and also, it is said, were added directly to bread either to aid in aeration or to counteract the effects of spoilage.

Distillation Method. Distil 5 g. of the sample, diluted to 250 ml. with a few milliliters of *sodium hydroxide solution* (baking powder) or 5 g. of *freshly calcined magnesium oxide* (vegetable and animal products) into *standard acid.* Titrate back with *standard alkali* as in the Willfarth modification.

Distillation of animal and vegetable products with the sodium hydroxide solution is not feasible, because of the decomposition of nitrogenous substances other than ammonium salts.

Aeration Methods. Aeration is believed to give more satisfactory results than distillation in the examination of foods for decomposition, where the amount of ammonia is small and the relative error due to decomposition of organic nitrogen is great. Modifications applicable to meat and eggs are described in Part II, H1 and 7.

f. Nitrates

See also Part II, H1.

Several volumetric and gasometric methods have been based on the following reaction:

$$3FeCl_2 \quad + 4HCl \rightarrow$$
$$3\ FeCl_3 + KCl + 2H_2O + NO$$

Schloesing-Schulze Nitric Oxide Gasometric Method. Schloesing[207] first collected the gas quantitatively, but without measuring its volume; Schulze[208] modified the process and calculated the results from the volume, correcting for temperature, atmospheric pressure, and tension of aqueous vapor. Tiemann,[209] working with Schulze, introduced minor changes, as did also Grandeau.[210]

The designation Schloesing-Schulze method seems preferable to Schulze-Tiemann method, as given by Fresenius-Cohn,[211] or Schloesing-Grandeau method modified by Tiemann and Schulze, as given by Treadwell-

The method is well suited for determining nitrates in condition powders and medicinal foods. As the accuracy is proportional to the quantity of nitrate in the solution, satisfactory results may be obtained in vegetable material such as beets (up to 0.2%) and cured tobacco (up to 0.7%).

Apparatus. The apparatus consists of (1) a 150-ml. *ring-neck flask* of strong glass to which is attached by means of a double-bored stopper a measuring tube with marks showing 15 and 20 ml. and a delivery tube, each with a middle section of best quality rubber tubing (with tied joints) and provided with a pinchcock, (2) a *gasometric tube,* and (3) a *crystallizing dish* which serves as a gas trough.

Process. *Expulsion of Air.* Place in the flask 25 ml. of a water extract or a dilute ethanol extract of a food, containing the nitrate, concentrated to about that volume if necessary. Add a piece of *paraffin* the size of a pea and, if a mineral acid is present, *sodium hydroxide solution* until slightly alkaline. Attach the cork and add sufficient water, by opening the pinchcock long enough to fill the measuring tube up to the expanded portion, then open the other pinchcock and slide it onto the glass tube. Heat cautiously to boiling and boil vigorously for 3 minutes to expel all air. While still boiling, adjust the

delivery tube beneath the mouth of the gasometric tube, which has previously been filled with *20% sodium hydroxide solution* and inverted in the crystallizing dish. An inverted buret calibrated to the cock may also be used.

Addition of Reagents. If the bubbles of steam are completely absorbed by the alkali, remove the lamp and at the moment boiling ceases, close the pinchcock on the delivery tube. Pour into the measuring tube 20 ml. of *ferrous chloride solution* (prepared by treating an excess of nails with hydrochloric acid), open the pinchcock, and close when nearly, but not quite, all the ferrous chloride solution has been sucked into the flask. Replace the lamp under the flask and add through the measuring tube as before 15 ml. of *hydrochloric acid.*

In introducing the liquids, it is imperative that no air be allowed to enter the flask.

Collection of Gas. Without delay—*this is the danger point*—ascertain whether the pressure in the flask is greater or less than that without. If the delivery tube is collapsed, it must be less; if it is not collapsed, open the pinchcock slightly every few seconds until the liquid in the tube moves outward, then open the pinchcock full, and boil until no more gas is given off.

The writers have made thousands of determinations by this method without a cut or burn, but an explosion is inevitable if the cock is not opened when the pressure is outward. It is well, however, to work in a hood with the sash partly down.

Reading. Remove the gasometric tube to a cylinder of water, holding a capsule by means of tongs under the mouth to prevent escape of liquid. When the temperature becomes constant, raise the tube so that the levels of the liquid within and without coincide and read the volume of gas. Note at the same time the temperature, the barometric pressure in millimeters, and the temperature of the barometer.

CALCULATION. Calculate the result by the following formulas in which V is milliliters of gas (corrected for errors in the graduation), G is grams of nitrogen in V, t is temperature of gas, b is barometric reading in millimeters, t' is temperature of barometer, w is tension of aqueous vapor at t, and b' is barometric reading calculated to 0° C.

$$b' = b - \qquad 5508 + t'$$

and

$$G \qquad \frac{(b' - w)0.0006256V}{760(1 + 0.00366t)}$$

The tension of aqueous vapor from 0 to 40° is given in the following table.[213]

Degrees C.	Tension mm.	Degrees C.	Tension mm.	Degrees C.	Tension mm.
0	4.53	14	11.88	28	28.15
1	4.87	15	12.68	29	29.83
2	5.23	16	13.52	30	31.60
3	5.62	17	14.41	31	33.46
4	6.03	18	15.35	32	35.42
5	6.47	19	16.35	33	37.47
6	6.94	20	17.40	34	39.63
7	7.44	21	18.51	35	41.89
8	7.96	22	19.68	36	44.27
9	8.53	23	20.91	37	46.76
10	9.13	24	22.21	38	49.37
11	9.75	25	23.58	39	52.10
12	10.42	26	25.03	40	54.97
13	11.13	27	26.55		

Special tables (such as Kohlmann and Frerich's), giving the weights of nitrogen corresponding to different temperatures and barometric pressures, or a graph may be used to facilitate the calculation.

For the small amounts of nitrates usually present in most food products, the barometric reading may be omitted and the average pressure for the region assumed.

NOTE. When a considerable number of determinations are made, the Schiff azotometer,[214] as modified by Johnson and Jenkins[215] (Fig. 56) may be substituted for the gasometric tube. The evolution flask and connecting tubes are the same as described except that a small perforated rubber stopper is so placed on the delivery tube that when it is introduced into the azotometer it brings the end of the tube in the proper position for delivering the gas. In inserting the tube, the bulb is slightly raised so that when the stopper is in position all air is excluded.

Ulsch Reduced Iron Volumetric Method.[216] The nitrate is reduced by *iron* to ammonium salt from which the ammonia is liberated by *sodium hydroxide solution*, distilled, and titrated.

Street Modification.[217] This modification, once official, no longer appears in the published methods of the A.O.A.C. It is here included since the Olsen method for total nitrogen and the Pucher, Leavenworth, and Vickery modification are combinations of this with the Gunning-Willfarth method.

PROCESS. Digest for a short time at room temperature 1 g. of the sample in a Kjeldahl flask together with 30 ml. of water and 2 to 3 g. of *reduced iron*, then add 10 ml. of *1 + 1 sulfuric acid*, shake well, and let stand until the reaction subsides. Heat slowly to boiling, continue the boiling for 5 minutes, cool, add 100 ml. of water, a little *paraffin*, and 7 to 10 g. of *magnesium oxide* practically free from carbonates, then distil into *standard acid* and titrate as in the determination of ammonia nitrogen.

CALCULATION. Subtract from the joint percentage of nitrogen from preformed ammonia and ammonia reduced from nitrate

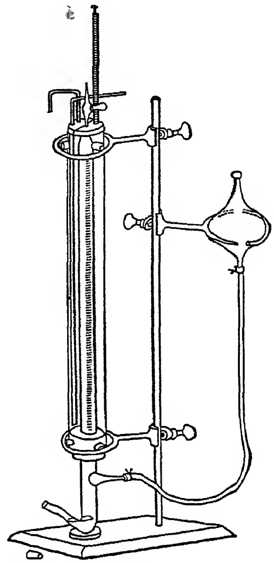


FIG. 56. Schiff Azotometer Modified by Johnson and Jenkins.

that from ammonia nitrogen separately determined. The difference is the percentage of nitrate nitrogen.

5. FAT OR OIL

(Ether Extract)

a. True Fat and Fixed Oil

See Part II, B2.

b. Volatile (Essential) Oil

See Part II, J1, 2, and 3.

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6. NIFEXT

(Nitrogen-Free Extract)

Although the constituents of the nitrogen-free extract do not represent a chemical group, the carbohydrates, organic acids, tannins, and other substances obtained by difference are conveniently included here.

a. Carbohydrates

Molisch α-Naphthol Test for Carbohydrates.[1] Mix 5 ml. of the solution with 1 or 2 drops of 10% ethanolic *α-naphthol solution,* then underlay with a small amount of *sulfuric acid.* In the presence of a soluble carbohydrate, a violet ring is formed at the zone of contact.

Fehling Copper Reduction Test. The quantitative determination of reducing sugar, in which a mixture of *copper sulfate* and *alkaline Rochelle salt solution* is employed, is treated below. The qualitative test is variously performed. In the clinical laboratory it is much used in urine analysis.

Barfoed Copper Acetate Test.[2] This test is useful in roughly distinguishing monosaccharides from reducing disaccharides.

Reagent. *Cupric Acetate Reagent.* Dissolve 50 g. of $Cu(CH_3CO_2)_2 \cdot H_2O$ in 750 ml. of water. For use, mix 200 ml. of this solution with 2 ml. of glacial acetic acid.

Process. Boil the solution of the unknown and add a small amount of the *reagent.* Hexoses readily reduce the solution with the formation of cuprous oxide; lactose and maltose do not to any considerable extent.

Benedict Copper Citrate Test.[3] Reagent. Prepare a solution of 17.3 g. of sodium citrate and 10 g. of Na_2CO_3 in 80 ml. of hot water. Add to the filtrate slowly with stirring a solution of 1.73 g. of $CuSO_4 \cdot 5H_2O$ in 10 ml. of water and filter.

Process. Heat to boiling 5 ml. of the *reagent* in a test tube, add 8 drops of the sugar solution, and boil for 1 or 2 minutes. The presence of reducing sugar is indicated by the formation on cooling of a voluminous greenish yellow or red precipitate.

Note. Although devised especially for detecting reducing sugars in urine, the test is also suited for various animal extracts containing creatine, creatinine, and other bases.

Thomas and Dutcher Modification of the Benedict and Osterberg Picrate-Picric Test.[4] The quantitative procedure described below may be used as a qualitative test.

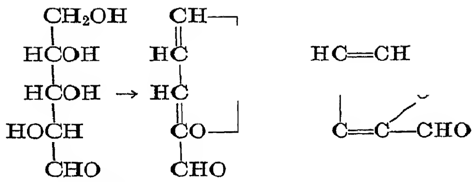
Phloroglucinol-Hydrochloric Acid Test. Pentoses and galactose react with the *reagent* with the formation of a cherry-red color. The quantitative determination of these sugars is described below.

Tollens Silver Test.[5] According to Browne and Zerban, who give full details, this is the most sensitive of the tests employing metallic salts. Its use is not recommended because of the explosive nature of the silver compounds formed.

Pentoses and Pentosans

As brought out by Tollens and his students, these polysaccharides have the general formula $(C_5H_8O_4)_n$ and pass, on hydrolysis by weak acid, into the pentose sugars, $C_5H_{10}O_5$. On boiling with concentrated acid, the pentoses are decomposed with the formation of furfural. The change is elucidated by considering that arabinose, a typical pentose, loses 3 molecules of water, as shown in the equation at the top of the next page.

The furfural, as first suggested by Councler,[6] is precipitated by phloroglucinol (phloroglucin), $C_6H_3(OH)_3 + 2H_2O$, as a



Arabinose Furfural

phloroglucide which may be separated by filtration, dried, and weighed. Since the precipitation is not complete, a correction is made for the amount remaining in solution, using the factor 0.0052 in the formulas below. The original method, described by Tollens and Krüger,[7] was brought to its present form by Kröber. It was introduced into the United States by Tollens' American students, Allen, Browne, de Chalmot, Lindsey, Ross, Stone, Washburn, and Wheeler.

Tollens and Kröber Phloroglucinol Gravimetric Method.[8] REAGENT. *Phloroglucinol Reagent.* Although phloroglucinol of suitable purity is now available, each lot should be tested by heating nearly to boiling a small quantity, dissolved in a few drops of acetic anhydride, and adding a few drops of H_2SO_4. A pronounced violet coloration indicates the presence of diresorcin in sufficient amount to vitiate the test. This impurity crystallizes out if the reagent (11 g. of phloroglucinol in 1500 ml. of 12% HCl) is prepared by first adding the phloroglucinol in small amounts with stirring to 300 ml. of the hot acid and then pouring this solution into the remainder of the acid which has not been heated, allowing this to stand at least overnight.

PROCESS. *Distillation with Acid.* Weigh out into a flask an amount of the sample believed sufficient to yield between 0.03 and 0.3 g. of furfural in the process, add 100 ml. of *12% hydrochloric acid* and a few pieces of *pumice stone* and connect with the distillation apparatus, by means of a double-bored rubber stopper, the second hole carrying a cylindrical separatory funnel with marks at 30 and 60 ml. Heat at first cautiously, then adjust the flame so that the distillate passing through a filter paper into a graduated cylinder will fill to the 30 ml. mark in about 10 minutes.

Determination as Phloroglucide. Repeat the distillation, adding 30 ml. of *12% hydrochloric acid* from the separatory funnel so manipulated as to wash down the sides of the flask, as many times (usually 9 to 12) as are necessary to remove all the furfural as shown by testing with analine-acetate paper. To the joint distillates, add the *phloroglucinol reagent* in about double the amount required to combine the furfural from the amount of pentosans judged to be present. The color of the solution changes first to yellow, then to green; then an amorphous greenish precipitate which changes rapidly to black forms. Dilute to 400 ml. with *12% hydrochloric acid,* allow to stand overnight, filter on a Gooch crucible, tared in a weighed bottle, wash with 150 ml. of water, keeping the crucible filled with the liquid until all has been added. Dry the phloroglucide on a water bath for 4 hours, cool in the weighing bottle, and weigh.

CALCULATION. If the weight of phloroglucide (a) is between 0.03 and 0.30 g., calculate by Kröber's table herewith or by the following formulas derived by Browne:

$$\text{Furfural} = (a + 0.0052) \times 0.5185 \text{ g.}$$
$$\text{Pentoses} = (a + 0.0052) \times 1.0075 \text{ g.}$$
$$\text{Pentosans} = (a + 0.0052) \times 0.8866 \text{ g.}$$

For weights outside the limits of the table, calculate the results by the following formulas:

For weight of phloroglucide a under 0.03 g.:

$$\text{Furfural} = (a + 0.0052) \times 0.5170 \text{ g.}$$
$$\text{Pentoses} = (a + 0.0052) \times 1.0170 \text{ g.}$$
$$\text{Pentosans} = (a + 0.0052) \times 0.8949 \text{ g.}$$

For weight of phloroglucide a over 0.30 g.:

$$\text{Furfural} = (a + 0.0052) \times 0.5180 \text{ g.}$$
$$\text{Pentoses} = (a + 0.0052) \times 1.0026 \text{ g.}$$
$$\text{Pentosans} = (a + 0.0052) \times 0.8824 \text{ g.}$$

PENTOSES AND PENTOSANS FROM PHLOROGLUCIDE (KRÖBER)

Phloroglucide	Furfural	Arabinose	Araban	Xylose	Xylan	Pentose	Pentosan
0.030	0.0182	0.0391	0.0344	0.0324	0.0285	0.0358	0.0315
.031	.0188	.0402	.0354	.0333	.0293	.0368	.0324
.032	.0193	.0413	.0363	.0342	.0301	.0378	.0333
.033	.0198	.0424	.0373	.0352	.0309	.0388	.0341
.034	.0203	.0435	.0383	.0361	.0317	.0398	.0350
.035	.0209	.0446	.0393	.0370	.0326	.0408	.0359
.036	.0214	.0457	.0402	.0379	.0334	.0418	.0368
.037	.0219	.0468	.0412	.0388	.0342	.0428	.0377
.038	.0224	.0479	.0422	.0398	.0350	.0439	.0386
.039	.0229	.0490	.0431	.0407	.0358	.0449	.0395
.040	.0235	.0501	.0441	.0416	.0366	.0459	.0404
.041	.0240	.0512	.0451	.0425	.0374	.0469	.0413
.042	.0245	.0523	.0460	.0434	.0382	.0479	.0422
.043	.0250	.0534	.0470	.0443	.0390	.0489	.0431
.044	.0255	.0545	.0480	.0452	.0398	.0499	.0440
.045	.0260	.0556	.0490	.0462	.0406	.0509	.0448
.046	.0266	.0567	.0499	.0471	.0414	.0519	.0457
.047	.0271	.0578	.0509	.0480	.0422	.0529	.0466
.048	.0276	.0589	.0519	.0489	.0430	.0539	.0475
.049	.0281	.0600	.0528	.0498	.0438	.0549	.0484
.050	.0286	.0611	.0538	.0507	.0446	.0559	.0492
.051	.0292	.0622	.0548	.0516	.0454	.0569	.0501
.052	.0297	.0633	.0557	.0525	.0462	.0579	.0510
.053	.0302	.0644	.0567	.0534	.0470	.0589	.0519
.054	.0307	.0655	.0576	.0543	.0478	.0599	.0528
.055	.0312	.0666	.0586	.0553	.0486	.0610	.0537
.056	.0318	.0677	.0596	.0562	.0494	.0620	.0546
.057	.0323	.0688	.0605	.0571	.0502	.0630	.0555
.058	.0328	.0699	.0615	.0580	.0510	.0640	.0564
.059	.0333	.0710	.0624	.0589	.0518	.0650	.0573
.060	.0338	.0721	.0634	.0598	.0526	.0660	.0581
.061	.0344	.0732	.0644	.0607	.0534	.0670	.0590
.062	.0349	.0743	.0653	.0616	.0542	.0680	.0599
.063	.0354	.0754	.0663	.0626	.0550	.0690	.0608
.064	.0359	.0765	.0673	.0635	.0558	.0700	.0617
.065	.0364	.0776	.0683	.0644	.0567	.0710	.0625
.066	.0370	.0787	.0692	.0653	.0575	.0720	.0634
.067	.0375	.0798	.0702	.0662	.0583	.0730	.0643
.068	.0380	.0809	.0712	.0672	.0591	.0741	.0652
.069	.0385	.0820	.0721	.0681	.0599	.0751	.0661

PENTOSES AND PENTOSANS FROM PHLOROGLUCIDE (KRÖBER)—*Continued*

Phloroglucide	Furfural	Arabinose	Araban	Xylose	Xylan	Pentose	Pentosan
0.070	0.0390	0.0831	0.0731	0.0690	0.0607	0.0761	0.0670
.071	.0396	.0842	.0741	.0699	.0615	.0771	.0679
.072	.0401	.0853	.0750	.0708	.0623	.0781	.0688
.073	.0406	.0864	.0760	.0717	.0631	.0791	.0697
.074	.0411	.0875	.0770	.0726	.0639	.0801	.0706
.075	.0416	.0886	.0780	.0736	.0647	.0811	.0714
.076	.0422	.0897	.0789	.0745	.0655	.0821	.0722
.077	.0427	.0908	.0799	.0754	.0663	.0831	.0731
.078	.0432	.0919	.0809	.0763	.0671	.0841	.0740
.079	.0437	.0930	.0818	.0772	.0679	.0851	.0749
.080	.0442	.0941	.0828	.0781	.0687	.0861	.0758
.081	.0448	.0952	.0838	.0790	.0695	.0871	.0767
.082	.0453	.0963	.0847	.0799	.0703	.0881	.0776
.083	.0458	.0974	.0857	.0808	.0711	.0891	.0785
.084	.0463	.0985	.0867	.0817	.0719	.0901	.0794
.085	.0468	.0996	.0877	.0827	.0727	.0912	.0803
.086	.0474	.1007	.0886	.0836	.0735	.0922	.0812
.087	.0479	.1018	.0896	.0845	.0743	.0932	.0821
.088	.0484	.1029	.0906	.0854	.0751	.0942	.0830
.089	.0489	.1040	.0915	.0863	.0759	.0952	.0838
.090	.0494	.1051	.0925	.0872	.0767	.0962	.0847
.091	.0499	.1062	.0935	.0881	.0775	.0972	.0856
.092	.0505	.1073	.0944	.0890	.0783	.0982	.0865
.093	.0510	.1084	.0954	.0900	.0791	.0992	.0874
.094	.0515	.1095	.0964	.0909	.0800	.1002	.0883
.095	.0520	.1106	.0974	.0918	.0808	.1012	.0891
.096	.0525	.1117	.0983	.0927	.0816	.1022	.0899
.097	.0531	.1128	.0993	.0936	.0824	.1032	.0908
.098	.0536	.1139	.1003	.0946	.0832	.1043	.0917
.099	.0541	.1150	.1012	.0955	.0840	.1053	.0926
.100	.0546	.1161	.1022	.0964	.0848	.1063	.0935
.101	.0551	.1171	.1032	.0973	.0856	.1073	.0944
.102	.0557	.1182	.1041	.0982	.0864	.1083	.0953
.103	.0562	.1193	.1051	.0991	.0872	.1093	.0962
.104	.0567	.1204	.1060	.1000	.0880	.1103	.0971
.105	.0572	.1215	.1070	.1010	.0888	.1113	.0976
.106	.0577	.1226	.1080	.1019	.0896	.1123	.0988
.107	.0582	.1237	.1089	.1028	.0904	.1133	.0997
.108	.0588	.1248	.1099	.1037	.0912	.1143	.1006
.109	.0593	.1259	.1108	.1046	.0920	.1153	.1015

PENTOSES AND PENTOSANS FROM PHLOROGLUCIDE (KRÖBER)—*Continued*

Phloroglucide	Furfural	Arabinose	Araban	Xylose	Xylan	Pentose	Pentosan
0.110	0.0598	0.1270	0.1118	0.1055	0.0928	0.1163	0.1023
.111	.0603	.1281	.1128	.1064	.0936	.1173	.1032
.112	.0608	.1292	.1137	.1073	.0944	.1183	.1041
.113	.0614	.1303	.1147	.1082	.0952	.1193	.1050
.114	.0619	.1314	.1156	.1091	.0960	.1203	.1059
.115	.0624	.1325	.1166	.1101	.0968	.1213	.1067
.116	.0629	.1336	.1176	.1110	.0976	.1223	.1076
.117	.0634	.1347	.1185	.1119	.0984	.1233	.1085
.118	.0640	.1358	.1195	.1128	.0992	.1243	.1094
.119	.0645	.1369	.1204	.1137	.1000	.1253	.1103
.120	.0650	.1380	.1214	.1146	.1008	.1263	.1111
.121	.0655	.1391	.1224	.1155	.1016	.1273	.1120
.122	.0660	.1402	.1233	.1164	.1024	.1283	.1129
.123	.0665	.1413	.1243	.1173	.1032	.1293	.1138
.124	.0671	.1424	.1253	.1182	.1040	.1303	.1147
.125	.0676	.1435	.1263	.1192	.1049	.1314	.1156
.126	.0681	.1446	.1272	.1201	.1057	.1324	.1165
.127	.0686	.1457	.1282	.1210	.1065	.1334	.1174
.128	.0691	.1468	.1292	.1219	.1073	.1344	.1183
.129	.0697	.1479	.1301	.1228	.1081	.1354	.1192
.130	.0702	.1490	.1311	.1237	.1089	.1364	.1201
.131	.0707	.1501	.1321	.1246	.1097	.1374	.1210
.132	.0712	.1512	.1330	.1255	.1105	.1384	.1219
.133	.0717	.1523	.1340	.1264	.1113	.1394	.1227
.134	.0723	.1534	.1350	.1273	.1121	.1404	.1236
.135	.0728	.1545	.1360	.1283	.1129	.1414	.1244
.136	.0733	.1556	.1369	.1292	.1137	.1424	.1253
.137	.0738	.1567	.1379	.1301	.1145	.1434	.1262
.138	.0743	.1578	.1389	.1310	.1153	.1444	.1271
.139	.0748	.1589	.1398	.1319	.1161	.1454	.1280
.140	.0754	.1600	.1408	.1328	.1169	.1464	.1288
.141	.0759	.1611	.1418	.1337	.1177	.1474	.1297
.142	.0764	.1622	.1427	.1346	.1185	.1484	.1306
.143	.0769	.1633	.1437	.1355	.1193	.1494	.1315
.144	.0774	.1644	.1447	.1364	.1201	.1504	.1324
.145	.0780	.1655	.1457	.1374	.1209	.1515	.1333
.146	.0785	.1666	.1466	.1383	.1217	.1525	.1342
.147	.0790	.1677	.1476	.1392	.1225	.1535	.1351
.148	.0795	.1688	.1486	.1401	.1233	.1545	.1360
.149	.0800	.1699	.1495	.1410	.1241	.1555	.1369

PENTOSES AND PENTOSANS FROM PHLOROGLUCIDE (KRÖBER)—*Continued*

Phloroglucide	Furfural	Arabinose	Araban	Xylose	Xylan	Pentose	Pentosan
0.150	0.0805	0.1710	0.1505	0.1419	0.1249	0.1565	0.1377
.151	.0811	.1721	.1515	.1428	.1257	.1575	.1386
.152	.0816	.1732	.1524	.1437	.1265	.1585	.1395
.153	.0821	.1743	.1534	.1446	.1273	.1595	.1404
.154	.0826	.1754	.1544	.1455	.1281	.1605	.1413
.155	.0831	.1765	.1554	.1465	.1289	.1615	.1421
.156	.0837	.1776	.1563	.1474	.1297	.1625	.1430
.157	.0842	.1787	.1573	.1483	.1305	.1635	.1439
.158	.0847	.1798	.1583	.1492	.1313	.1645	.1448
.159	.0852	.1809	.1592	.1501	.1321	.1655	.1457
.160	.0857	.1820	.1602	.1510	.1329	.1665	.1465
.161	.0863	.1831	.1612	.1519	.1337	.1675	.1474
.162	.0868	.1842	.1621	.1528	.1345	.1685	.1483
.163	.0873	.1853	.1631	.1537	.1353	.1695	.1492
.164	.0878	.1864	.1640	.1546	.1361	.1705	.1501
.165	.0883	.1875	.1650	.1556	.1369	.1716	.1510
.166	.0888	.1886	.1660	.1565	.1377	.1726	.1519
.167	.0894	.1897	.1669	.1574	.1385	.1736	.1528
.168	.0899	.1908	.1679	.1583	.1393	.1746	.1537
.169	.0904	.1919	.1688	.1592	.1401	.1756	.1546
.170	.0909	.1930	.1698	.1601	.1409	.1766	.1554
.171	.0914	.1941	.1708	.1610	.1417	.1776	.1563
.172	.0920	.1952	.1717	.1619	.1425	.1786	.1572
.173	.0925	.1963	.1727	.1628	.1433	.1796	.1581
.174	.0930	.1974	.1736	.1637	.1441	.1806	.1590
.175	.0935	.1985	.1746	.1647	.1449	.1816	.1598
.176	.0940	.1996	.1756	.1656	.1457	.1826	.1607
.177	.0946	.2007	.1765	.1665	.1465	.1836	.1616
.178	.0951	.2018	.1775	.1674	.1473	.1846	.1625
.179	.0956	.2029	.1784	.1683	.1481	.1856	.1634
.180	.0961	.2039	.1794	.1692	.1489	.1866	.1642
.181	.0966	.2050	.1804	.1701	.1497	.1876	.1651
.182	.0971	.2061	.1813	.1710	.1505	.1886	.1660
.183	.0977	.2072	.1823	.1719	.1513	.1896	.1669
.184	.0982	.2082	.1832	.1728	.1521	.1906	.1678
.185	.0987	.2093	.1842	.1738	.1529	.1916	.1686
.186	.0992	.2104	.1851	.1747	.1537	.1926	.1695
.187	.0997	.2115	.1861	.1756	.1545	.1936	.1704
.188	.1003	.2126	.1870	.1765	.1553	.1946	.1712
.189	.1008	.2136	.1880	.1774	.1561	.1955	.1721

PENTOSES AND PENTOSANS FROM PHLOROGLUCIDE (KRÖBER)—*Continued*

Phloroglucide	Furfural	Arabinose	Araban	Xylose	Xylan	Pentose	Pentosan
0.190	0.1013	0.2147	0.1889	0.1783	0.1569	0.1965	0.1729
.191	.1018	.2158	.1899	.1792	.1577	.1975	.1738
.192	.1023	.2168	.1908	.1801	.1585	.1985	.1747
.193	.1028	.2179	.1918	.1810	.1593	.1995	.1756
.194	.1034	.2190	.1927	.1819	.1601	.2005	.1764
.195	.1039	.2201	.1937	.1829	.1609	.2015	.1773
.196	.1044	.2212	.1946	.1838	.1617	.2025	.1782
.197	.1049	.2222	.1956	.1847	.1625	.2035	.1791
.198	.1054	.2233	.1965	.1856	.1633	.2045	.1800
.199	.1059	.2244	.1975	.1865	.1641	.2055	.1808
.200	.1065	.2255	.1984	.1874	.1649	.2065	.1817
.201	.1070	.2266	.1994	.1883	.1657	.2075	.1826
.202	.1075	.2276	.2003	.1892	.1665	.2085	.1835
.203	.1080	.2287	.2013	.1901	.1673	.2095	.1844
.204	.1085	.2298	.2022	.1910	.1681	.2105	.1853
.205	.1090	.2309	.2032	.1920	.1689	.2115	.1861
.206	.1096	.2320	.2041	.1929	.1697	.2125	.1869
.207	.1101	.2330	.2051	.1938	.1705	.2134	.1878
.208	.1106	.2341	.2060	.1947	.1713	.2144	.1887
.209	.1111	.2352	.2069	.1956	.1721	.2154	.1896
.210	.1116	.2363	.2079	.1965	.1729	.2164	.1904
.211	.1121	.2374	.2089	.1975	.1737	.2174	.1913
.212	.1127	.2384	.2098	.1984	.1745	.2184	.1922
.213	.1132	.2395	.2108	.1993	.1753	.2194	.1931
.214	.1137	.2406	.2117	.2002	.1761	.2204	.1940
.215	.1142	.2417	.2127	.2011	.1770	.2214	.1948
.216	.1147	.2428	.2136	.2020	.1778	.2224	.1957
.217	.1152	.2438	.2146	.2029	.1786	.2234	.1966
.218	.1158	.2449	.2155	.2038	.1794	.2244	.1974
.219	.1163	.2460	.2165	.2047	.1802	.2254	.1983
.220	.1168	.2471	.2174	.2057	.1810	.2264	.1992
.221	.1173	.2482	.2184	.2066	.1818	.2274	.2001
.222	.1178	.2492	.2193	.2075	.1826	.2284	.2010
.223	.1183	.2503	.2203	.2084	.1834	.2294	.2019
.224	.1189	.2514	.2212	.2093	.1842	.2304	.2028
.225	.1194	.2525	.2222	.2102	.1850	.2314	.2037
.226	.1199	.2536	.2232	.2111	.1858	.2324	.2046
.227	.1204	.2546	.2241	.2121	.1866	.2334	.2054
.228	.1209	.2557	.2251	.2130	.1874	.2344	.2063
.229	.1214	.2568	.2260	.2139	.1882	.2354	.2072

PENTOSES AND PENTOSANS FROM PHLOROGLUCIDE (KRÖBER)—*Continued*

Phloroglucide	Furfural	Arabinose	Araban	Xylose	Xylan	Pentose	Pentosan
0.230	0.1220	0.2579	0.2270	0.2148	0.1890	0.2364	0.2081
.231	.1225	.2590	.2280	.2157	.1898	.2374	.2089
.232	.1230	.2600	.2289	.2166	.1906	.2383	.2097
.233	.1235	.2611	.2299	.2175	.1914	.2393	.2106
.234	.1240	.2622	.2308	.2184	.1922	.2403	.2115
.235	.1245	.2633	.2318	.2193	.1930	.2413	.2124
.236	.1251	.2644	.2327	.2202	.1938	.2423	.2132
.237	.1256	.2654	.2337	.2211	.1946	.2433	.2141
.238	.1261	.2665	.2346	.2220	.1954	.2443	.2150
.239	.1266	.2676	.2356	.2229	.1962	.2453	.2159
.240	.1271	.2687	.2365	.2239	.1970	.2463	.2168
.241	.1276	.2698	.2375	.2248	.1978	.2473	.2176
.242	.1281	.2708	.2384	.2257	.1986	.2483	.2185
.243	.1287	.2719	.2394	.2266	.1994	.2493	.2194
.244	.1292	.2730	.2403	.2275	.2002	.2503	.2203
.245	.1297	.2741	.2413	.2284	.2010	.2513	.2212
.246	.1302	.2752	.2422	.2293	.2018	.2523	.2220
.247	.1307	.2762	.2432	.2302	.2026	.2533	.2229
.248	.1312	.2773	.2441	.2311	.2034	.2543	.2238
.249	.1318	.2784	.2451	.2320	.2042	.2553	.2247
.250	.1323	.2795	.2460	.2330	.2050	.2563	.2256
.251	.1328	.2806	.2470	.2339	.2058	.2573	.2264
.252	.1333	.2816	.2479	.2348	.2066	.2582	.2272
.253	.1338	.2827	.2489	.2357	.2074	.2592	.2281
.254	.1343	.2838	.2498	.2366	.2082	.2602	.2290
.255	.1349	.2849	.2508	.2375	.2090	.2612	.2299
.256	.1354	.2860	.2517	.2384	.2098	.2622	.2307
.257	.1359	.2870	.2526	.2393	.2106	.2632	.2316
.258	.1364	.2881	.2536	.2402	.2114	.2642	.2325
.259	.1369	.2892	.2545	.2411	.2122	.2652	.2334
.260	.1374	.2903	.2555	.2420	.2130	.2662	.2343
.261	.1380	.2914	.2565	.2429	.2138	.2672	.2351
.262	.1385	.2924	.2574	.2438	.2146	.2681	.2359
.263	.1390	.2935	.2584	.2447	.2154	.2691	.2368
.264	.1395	.2946	.2593	.2456	.2162	.2701	.2377
.265	.1400	.2957	.2603	.2465	.2170	.2711	.2385
.266	.1405	.2968	.2612	.2474	.2178	.2721	.2394
.267	.1411	.2978	.2622	.2483	.2186	.2731	.2403
.268	.1416	.2989	.2631	.2492	.2194	.2741	.2412
.269	.1421	.3000	.2641	.2502	.2202	.2751	.2421

PENTOSES AND PENTOSANS FROM PHLOROGLUCIDE (KRÖBER)—*Concluded*

Phloroglucide	Furfural	Arabinose	Araban	Xylose	Xylan	Pentose	Pentosan
0.270	0.1426	0.3011	0.2650	0.2511	0.2210	0.2761	0.2429
.271	.1431	.3022	.2660	.2520	.2218	.2771	.2438
.272	.1436	.3032	.2669	.2529	.2226	.2781	.2447
.273	.1442	.3043	.2679	.2538	.2234	.2791	.2456
.274	.1447	.3054	.2688	.2547	.2242	.2801	.2465
.275	.1452	.3065	.2698	.2556	.2250	.2811	.2473
.276	.1457	.3076	.2707	.2565	.2258	.2821	.2482
.277	.1462	.3086	.2717	.2574	.2266	.2830	.2490
.278	.1467	.3097	.2726	.2583	.2274	.2840	.2499
.279	.1473	.3108	.2736	.2592	.2282	.2850	.2508
.280	.1478	.3119	.2745	.2602	.2290	.2861	.2517
.281	.1483	.3130	.2755	.2611	.2298	.2871	.2526
.282	.1488	.3140	.2764	.2620	.2306	.2880	.2534
.283	.1493	.3151	.2774	.2629	.2314	.2890	.2543
.284	.1498	.3162	.2783	.2638	.2322	.2900	.2552
.285	.1504	.3173	.2793	.2647	.2330	.2910	.2561
.286	.1509	.3184	.2802	.2656	.2338	.2920	.2570
.287	.1514	.3194	.2812	.2665	.2346	.2930	.2578
.288	.1519	.3205	.2821	.2674	.2354	.2940	.2587
.289	.1524	.3216	.2831	.2683	.2362	.2950	.2596
.290	.1529	.3227	.2840	.2693	.2370	.2960	.2605
.291	.1535	.3238	.2850	.2702	.2378	.2970	.2614
.292	.1540	.3248	.2859	.2711	.2386	.2980	.2622
.293	.1545	.3259	.2868	.2720	.2394	.2990	.2631
.294	.1550	.3270	.2878	.2729	.2402	.3000	.2640
.295	.1555	.3281	.2887	.2738	.2410	.3010	.2649
.296	.1560	.3292	.2897	.2747	.2418	.3020	.2658
.297	.1566	.3302	.2906	.2756	.2426	.3030	.2666
.298	.1571	.3313	.2916	.2765	.2434	.3040	.2675
.299	.1576	.3324	.2925	.2774	.2442	.3050	.2684
.300	.1581	.3335	.2935	.2784	.2450	.3060	.2693

NOTES. In experiments conducted by Sakostschikoff, Iwanowa, and Kurennowa,[9] it was found that the Tollens method yields results on cottonseed hulls that are only 47% of the true amount. In order to obtain the full content it is necessary to add the results of three determinations made on (1) the water extract after precipitation of tannins, (2) the acid extract, and (3) the residue after hydrolysis with acid.

Klingstedt [10] has demonstrated that, in the presence of considerable amounts of hexose or substances yielding hexose on hydrolysis, the method is inaccurate owing to the formation of hydroxymethylfuraldehyde. In order to obtain approximately satisfactory results

the distillation should be stopped when the pentosans are decomposed, which is usually when 150 to 180 ml. of distillate have been collected, and the progress of the distillation should be followed by the phloroglucinol-hydrochloric acid test. If the distillate contains more than a trace of furaldehyde, it has a greenish cast.

I. Khristich Copper Reduction Modification.[11] It is claimed that the modification shortens the time for determination to 2 hours with an accuracy of 0.1 to 1%.

PROCESS. Hydrolyze 2 g. of the sample with *hydrochloric acid* by the Tollens and Kröber method and treat the hydrolyzate with *calcium carbonate* and *sodium bicarbonate* until the liberation of carbon dioxide ceases, then add 20 ml. of *Fehling solution* and proceed as in the determination of reducing sugars by the Bertrand Method below. Collect the copper suboxide on a Gooch crucible or filtering tube, wash, dissolve in 20 to 25 ml. of *ferric sulfate solution* (50 g. with 200 g. of sulfuric acid per liter), and titrate with *0.1 N sodium permanganate solution* to a pink color. Since equal parts by weight of dextrose and furfural give the same amount of copper suboxide, Bertrand's table may be used.

II. Jayme and Sarten Hydrobromic Acid Modification.[12] As shown by Lechner and Illig,[13] hydrochloric acid methods are inaccurate. If the distillation is carried out with hydrobromic acid, the distillate need not be neutralized, furfural is not decomposed, and no by-products are formed.

PROCESS. Mix the charge in a 500-ml. flask with 150 ml. of *20 to 30% hydrobromic acid* and distil over 50 ml. of liquid at 104 to 105°. Add 50 ml. of water to the flask and continue the distillation with the further addition of water until in 2 to 4 hours 400 to 800 ml. have been collected. Precipitate the furfural with *barbituric acid* or *thiobarbituric acid*. Allow for the solubility of the precipitate, as shown by a curve plotted from results on pure furfural. Dextrose does not interfere since it does not form hydroxymethylfurfural.

Pervier and Gortner Bromate Electrical Titration Method.[14] These authors (University of Minnesota), recognizing that in the A.O.A.C. method the acid has considerable destructive effect on the furfural, propose carrying off the furfural as formed by a slow current of steam. They titrate the furfural with potassium bromate solution in an acid solution containing potassium bromide.

APPARATUS. *Steam Distillation Assembly. Electric Titration Apparatus*, consisting of a galvanometer, a tapping key, and two platinum wires, one sealed into the side of a small pipet and held at a fixed potential by immersion in an acidified solution of 20% potassium bromide containing a trace of free bromine, the other immersed in the solution of the unknown. The potential depends on the composition of the unknown which is mixed by electric stirring.

Before titration, depressing the key causes the deflection of the needle to the right or left, whereas after titration the excess of bromine produces a movement in the opposite direction. When the depression of the key causes no deflection, the end-point has been reached.

PROCESS. *Acid Distillation of Furfural.* Place 0.2 to 5.0 g. of the sample in a 750-ml. flask of the steam distillation assembly, add 200 ml. of *12% hydrochloric acid* (sp.gr. 1.06), and conduct a slow stream of steam into the mixture. As soon as the boiling point has been reached, heat with a low flame so that the boiling temperature remains between 103 and 105°, as shown by a thermometer in the neck. If care is taken, the need of extra acid is avoided. Continue the distillation until a drop of the distillate no longer gives a red coloration with *aniline test paper* on standing 3 to 5 minutes.

Bromate Titration. To the distillate containing 0.1 to 0.2 g. of furfural add 5 ml. of

20% potassium bromide solution for each 100 ml. and adjust the acidity to about 4% by adding either *hydrochloric acid* or *alkali*. Run in *standard 0.1 N potassium bromate solution* from a buret at such a rate as to avoid the formation of a distinct yellow coloration throughout the solution. When the approach to the end-point is indicated by the formation, immediately after the addition of a few drops of bromate solution, of a pale color that soon fades, add the *bromate solution* in 0.2- to 0.3-ml. increments and note the time required for the disappearance of the free bromine by the use of a galvanometer. When the end-point is crossed, as indicated by a rather large increase in the time required, carry the observation slightly further. Finally plot the ratio of time increment to the increment of standard potassium bromate against the total value of potassium bromate already used and find the end-point from the curve.

CALCULATION. Multiply by 0.004803 the number of milliliters of 0.1 *N* potassium bromate solution required to obtain the grams of furfural in the sample.

Powell and Whittaker Thiosulfate Titration Modification.[15] The procedure (Royal Arsenal, Woolwich) depends on the reaction in hydrochloric acid between 4 atoms of bromine and 1 molecule of furfural formed by the distillation with acid.

APPARATUS. *Distillation Assembly,* with glass joints.

REAGENT. *Standard Bromide-Bromate Solution,* 0.1 *N*. The authors do not state whether the normality given applies to the bromide as well as the bromate. The separate addition of the bromide, as directed by Pervier and Gortner, seems preferable.

PROCESS. *Distillation.* Weigh 0.5 to 0.8 g. of the sample into the distilling flask, add *12% hydrochloric acid* and distil in the usual manner until all the furfural has been removed. Dilute the distillate with *12% hydrochloric acid* to 500 ml. in a volumetric flask.

Bromate Treatment. Pipet 25 ml. of *standard 0.1 N sodium bromide-bromate solution* into each of 4 glass-stoppered flasks. Add to each of two of the flasks 200 ml. of the furfural distillate and to each of the other two 200 ml. of *12% hydrochloric acid* for controls. Stopper tightly and let stand in the dark for 1 hour.

Titration. Add to each flask 10 ml. of *10% potassium iodide solution* and titrate the liberated iodine with standard *0.1 N sodium thiosulfate solution.*

CALCULATION. Subtract the average number of milliliters of standard solution required by the sample from that required by the control and multiply the difference by 0.0024, thus obtaining the number of grams of furfural present in the 200-ml. aliquot of the distillate.

EXAMPLES. Although the method was designed for wood pulps, it may be applied to other lignified tissues. Results agreeing with those by the gravimetric phloroglucinol method are reported. (See Tollens and Kröber Phloroglucinol Gravimetric Method above.)

NOTE. Krishnan[16] found the method satisfactory if the temperature is properly controlled. At 30° the absorption of the bromine is proportional to the furfural irrespective of the bromine concentration. It is applicable to small amounts of furfural whereas the Phloroglucinol method is not.

Reeves and Munro Immiscible Solvent Colorimetric Method.[17] Instead of the mixture of the sample with hydrochloric acid being distilled, it is refluxed and the furfural in the sample is determined colorimetrically (Boyce Thompson Institute).

APPARATUS. *Refluxing Assembly,* with 50-ml. flask.

Colorimeter. Bausch & Lomb, Duboscq type.

REAGENTS. *Xylene,* redistilled if on long boiling with acid a precipitate develops. To avoid the harmful effects of xylene use a

rubber bulb in pipetting and protect the lungs against the vapors.

Standard Furfural Solution, 0.1%. Prepare the stock solution from redistilled furfural. If stored in the refrigerator, the solution keeps 10 months. Dilute the stock solution to prepare a series containing 0.01 to 0.025 mg. per ml.

Aniline Acetate Reagent. Dissolve 1 ml. of colorless aniline in 50 ml. of glacial acetic acid and 50 ml. of ethanol. Discard after 2 or 3 days when a yellow color develops.

PROCESS. *Xylene Extraction*. Place in the reaction flask a portion of the sample containing 0.4 to 1.0 mg. of pentose and 3 to 10 ml. of *1 + 2 hydrochloric acid*. Add 25 ml. of *xylene* and a small glass ebullition tube, then reflux 2.5 hours, boiling at a moderate rate. Cool to room temperature, decant the xylene layer, stir with a little *anhydrous sodium acetate* to remove moisture and mineral acid, and filter. If the solution is colorless, proceed with the color formation.

Distillation. If, owing to the presence of 10 parts or more of hexose, the solution has a brown coloration, distil to dryness *in vacuo* exactly 20 ml. contained in a 125-ml. Claisen flask, heating in a water bath and collecting the distillate in a well-cooled receiver. Add 3 ml. of *xylene* to the flask and distil as before. Transfer the distillate to a 25-ml. volumetric flask, using 2 ml. of *xylene* for rinsing, and dilute to the mark with *xylene*.

Color Formation. Place in one of two test tubes 5 ml. of the unknown and in the other 5 ml. of a *standard* differing from the unknown by no more than 25%. Add quickly to each exactly 5 ml. of *aniline acetate reagent* and allow to stand for 20 minutes in a dark place for the development of the red color.

Color Comparison. Make the comparison as rapidly as possible because the color is photosensitive.

CALCULATION. In making the calculation take account of the dilution of the unknown from 20 to 25 ml. if it was found necessary to remove a brown coloration.

PENTOSES AND METHYL PENTOSES

Tollens and Ellett Ethanol Gravimetric Method.[18] PROCESS. Place the Gooch crucible containing the weighed mixture of furfural and methyl furfural phloroglucides, obtained by the Tollens and Kröber phloroglucinol method for pentoses, in a small beaker together with *ethanol* and heat nearly to boiling. Remove the liquid from the crucible by suction and wash twice with hot *ethanol*, dry 2 hours in a boiling water oven, and weigh.

CALCULATION. Calculate the residue as pentose (or pentosan) as usual and the loss in weight as methyl pentose (or methyl pentosan) by the use of the following formulas, in which a is the weight of methylfurfural phloroglucide:

$$\text{Fucose} = 2.66a - 12.25a^2 + 0.0005$$
$$\text{Fucosan} = \text{fucose} \times 0.89$$
$$\text{Rhamnose} = 1.65a^2 - 1.84a^2 + 0.0100$$
$$\text{Rhamnosan} = \text{rhamnose} \times 0.8$$
$$\text{Methyl pentosan} = 1.85a - 6.25a^2 + 0.0040$$

NOTE. Haywood[19] places the crucible containing the mixed phloroglucides in a beaker, pours into the crucible 30 ml. of *ethanol* at 60°, and heats at 60° for 10 minutes, then sucks off the liquid, repeating the treatment three to five times and finally drying as above. Proceeding in this manner, he introduces a correction of 0.0037 g. for furfural phloroglucide dissolved by the ethanol.

METHYL PENTOSES

Nicolet and Shinn Periodic Acid Volumetric Method.[20] The method was developed in the U. S. Bureau of Dairy Industry. Methyl pentoses are the only sugars which form acetaldehyde when treated with periodic acid.

Apparatus. *Three Pyrex test tubes* (2.5 x 20 cm.) forming a gas absorption train. The first tube, in which acetaldehyde is generated, carries a dropping funnel, the stem of which reaches nearly to the bottom; the other two are arranged for the absorption of the gas.

Reagents. *Sodium Arsenite Solution*, about 0.1 N, in 2% sodium bicarbonate solution.

Sodium Bisulfite Solution, 2%, in 1.9% meta-bisulfite solution.

Process. *Preparation of Tubes.* In tube I place (1) the sample containing preferably 5 to 15 mg. of methyl pentoses in little over 5 ml., (2) about 0.2 g. of *alanine*, (3) 1 drop of *Nujol* to prevent foaming, unless the sample has been decolorized for the determination of other carbohydrates, (4) 5 ml. (10 ml. or more if large amounts of other carbohydrates are present) of *1.0 N sodium bicarbonate solution*, and (5) 10 ml. of *0.1 N sodium arsenite solution* which must be sufficient to reduce all the periodate not reduced by reaction with the sugars.

In tubes II and III place respectively 5 ml. and 3 ml. of *2% sodium bisulfite solution* diluted to 25 ml.

Treatment with Periodic Acid. Pass *carbon dioxide* through the train for several seconds to mix the contents of the tubes, then break the connection to introduce into the funnel tube (with stopcock closed) 1 or 2 ml. of *periodic acid* or an amount determined as follows:

Neutralize to *litmus* a portion of the sample with *sodium bicarbonate solution* and add 10 ml. additional, followed by 2 ml. of *0.5 M periodic acid.* Mix, allow to stand for 2 minutes, then add 2 ml. of *20% potassium iodide solution*, and titrate the liberated iodine with *standard arsenite solution*, the amount of which is equivalent, mole for mole, to the periodic acid excess. If there is no excess, start afresh with more periodate. Calculate the amount necessary for the actual analysis which must be (1) at least twice the periodate rapidly consumed by the solution itself and (2) at least enough arsenite to react with the excess of periodate. With these conditions met, moderate excesses of either reagent are not detrimental.

Reconnect the system, open the stopcock, and allow the *periodic acid solution* to enter under *carbon dioxide* pressure, thus guarding against loss of acetaldehyde and contamination from the air, as well as facilitating introduction of the reagent against back pressure.

Titration. After the carbon dioxide has passed through the train for 1 hour at about 1 liter per minute, mix the contents of tubes II and III, and titrate in the manner followed by Clausen [21] for the determination of lactic acid (which see), using, however, *0.02 N (not 0.002 N) iodine solution.*[21]

Calculation. Use: 1 ml. of 0.02 N iodine solution = 1.64 mg. of methyl pentose.

Note. If other carbohydrates are to be determined, treat the solution remaining in tube I as directed by Nicolet and Shinn for the determination of serine, under C4*b* above.

Galactose and Galactan

The determination of galactan and its sugar is of importance in plant chemistry.

Tollens and Rischbiet Mucic Acid Gravimetric Method.[22] This method, although used the world over, has been on probation for a generation in the United States. It depends on the theoretical formation of one molecule of mucic acid ($C_6H_{10}O_8$) from one molecule of galactan $(C_6H_{10}O_5)_n$ or galactose ($C_6H_{12}O_6$), although the complete reaction does not take place. A full discussion of the shortcomings of the method is given by Browne and Zerban.[23]

Reagents. *Ammonia-Ammonium Carbonate Solution.* Dissolve 1 part of ammonium carbonate in 19 parts of water and 1 part of NH_4OH.

Process. *Extraction.* Remove the fat from a 2.5- to 3.0-g. charge by extraction on a

hardened filter with 5 successive portions of 10 ml. of *ether*. Transfer the extracted residue to a beaker about 5.5 cm. in diameter and 7 cm. deep.

Formation of Mucic Acid. Add to the extracted residue 60 ml. of *25% nitric acid* (sp. gr. 1.15) and evaporate on the steam bath to 20 ml. After 24 hours add 10 ml. of water and allow to stand 24 hours longer, then collect the crude mucic acid crystals on a filter and wash with 30 ml. of water to remove the greater part of the nitric acid.

Formation of Ammonium Mucate. Return the filter and contents to the beaker, add 30 ml. of *ammonia-ammonium carbonate solution*, and heat in a water bath at 80° with constant stirring for 15 minutes, thus causing the ammonium carbonate to combine with the mucic acid, forming soluble ammonium mucate. Wash the filter and contents several times with hot water by decantation, passing the washings through a filter paper, and finally transfer the residue to the paper. Wash thoroughly on the paper and evaporate the filtrate and washings on the steam bath to dryness, avoiding overdrying and consequent decomposition.

Reprecipitation of Mucic Acid. Add 5 ml. of *25% nitric acid* to the residue, stir well, and allow to stand 30 minutes. Collect the precipitated mucic acid on a weighed Gooch crucible and wash with 10 to 15 ml. of water followed by 60 ml. of *ethanol* and by several portions of *ether*. Dry in a boiling water oven for 3 hours, cool in a desiccator, and weigh.

CALCULATION. Multiply by 1.33 and 1.20 to convert the weight of mucic acid into galactose and galactan respectively. These factors are empirical, since the yield of mucic acid falls far short of the theoretical.

SUCROSE

The methods are partly polariscopic and partly calculation, depending on conversion of specific gravity and refractive index into sucrose; they are described together with polariscopic technique in Part II, E2.

REDUCING SUGARS

(Dextrose, Levulose, Invert Sugar, Maltose, and Lactose by Copper Reduction Methods)

Since most of the methods are of general application (cereal products, fruit products, dairy products, spices, etc.), rather than limited to the analysis of saccharine products, they are described in this section.

Historical. The name of Fehling (1812–1885), a professor in the Stuttgart School of Technology, is associated with the copper reduction reagents as intimately as that of Clerget is with polarimetric sugar calculations. Browne,[24] who has delved into history, credits Trommer [25] with adapting reduction by alkaline copper sulfate solution to qualitative tests and Barreswil [26] with adding potassium tartrate to the reagent to prevent the precipitation of cuprous hydroxide. Fehling,[27] however, was the first to proportion properly the ingredients and place the method on a solid analytical foundation.

Soxhlet [28] (born 1848), a professor in the Munich School of Technology and director of the Bavarian Agricultural Experiment Station, brought out that the weight of cuprous oxide formed in the reduction is not strictly proportional to the sugar content, hence the need of a preliminary test to ascertain the approximate sugar content before making the actual volumetric analysis in a definite concentration of the sugar, or of determining the weight of copper reduced at progressive concentrations and from these data deriving a calculation formula or, by interpolation, a table. More recently Lane and Eynon have prepared calculation tables for use in connection with a volumetric conventional method.

The Bertrand volumetric method is essentially a modified copper reduction method, differing from the above in that the copper

precipitate is collected on a filter, dissolved in acid ferric sulfate solution, and titrated with permanganate solution. Examples of lesser used volumetric methods depending on reactions other than copper reduction are the Romijn iodometric method and its modifications and the Gentele ferricyanide method.

In gravimetric copper reduction methods the weight of the sugar, corresponding to the weight of copper, cuprous oxide, or cupric oxide, is obtained from a calculation table such as Munson and Walker's so-called unified table for dextrose, invert sugar, lactose, and maltose, Allihn's for dextrose, Soxhlet and Wein's for lactose, and Meissl's for invert sugar.

In using formulas and tables, strict adherence to the analytical procedure of the originators is imperative.

Fehling Solution. The solution, as prepared by Fehling, contained the same percentage of copper sulfate and sodium hydroxide as that used today, but the tartrate was potassium tartrate, not Rochelle salt; furthermore the three ingredients were all dissolved in the same solution, not in two solutions kept separate until needed.

Fehling-Soxhlet Reagent. 1. Dissolve 34.639 g. of crystallized copper sulfate ($CuSO_4 \cdot 5H_2O$), of highest purity and theoretical water content, in water and make up accurately to 500 ml. in a volumetric flask. Soxhlet recrystallized the salt once from a dilute nitric acid solution and three times from water, but a reagent grade is now available that needs only one recrystallization.

2. Dissolve 173 g. of recrystallized Rochelle salt (sodium potassium tartrate) and 50 g. of sodium hydroxide (by sodium) in water and make up to 500 ml. in a volumetric flask.

As needed, pipet the same amount (depending on the method) of 1 and 2 into the beaker or tube used for the determination.

Volumetric Copper Reduction Methods. Of the unified methods, based on the original Fehling process, two have been selected: first, the Soxhlet method, one of the oldest, because it is still extensively followed on the Continent and, second, the Lane and Eynon method, one of the most recent, because it introduces corrections for the errors of other volumetric methods.

Soxhlet Unified Copper Reduction Volumetric Method. REAGENTS. The two component *Fehling-Soxhlet solutions* prepared as above described.

PROCESS. *Preliminary Titration.* To a mixture of 5 ml. each of *solutions 1 and 2* and 30 ml. of water, contained in a porcelain casserole, add little by little from a buret the solution of the unknown, heating after each addition, 2 minutes for dextrose, fructose, and invert sugar, 4 minutes for maltose, and 6 minutes for lactose. Continue the alternate addition and heating until the solution over the red precipitate is practically colorless.

Multiply the number of milliliters of sugar solution added by the following factors to obtain the approximate weight in milligrams of sugars present: dextrose 4.753, levulose 5.144, invert sugar 4.941, galactose 5.110, crystalline lactose ($C_{12}H_{22}O_{11} \cdot H_2O$) 6.757, anhydrous lactose 6.419, and maltose 7.780.

Final Titration. From the result as above described dilute the solution of the sample until it contains no more than 1% of the sugar known to be present. Heat in a porcelain casserole a mixture of 25 ml. each of *solutions 1 and 2* and the calculated corresponding amount of the sugar solution added in one portion and note the color of the supernatant liquid. If blue or green, showing an excess of copper, the test must be repeated; if yellow, filter a portion through a moist filter, add *acetic acid* to acid reaction, and test with *potassium ferrocyanide solution.* Repeat the test, using more or less of the solution, until two determinations, one giving a positive, the other a negative reaction for copper, agree within 0.1 ml. An average of these two readings is accepted as final.

NOTE. Wiley [29] removes the test portion of the solution by sucking it up into a glass tube through an asbestos-coated filter cap on the end. Ross [30] dips a small pleated filter held by forceps into the solution and collects enough of the liquid for the test.

Bertrand Unified Copper Reduction Ferric-Permanganate Volumetric Method.[31] In France and Belgium, as well as in their sugar-growing colonies, this method is widely used. In English-speaking countries it also has its advocates. Kertész [32] has rearranged the Bertrand table and extended it so as to include additional sugars. Browne and Zerban [33] give the Kertész table in condensed form. The volumetric permanganate method for the determination of reduced cuprous oxide, adopted by the A.O.A.C., is essentially the Bertrand method beginning with the ferric sulfate treatment.

The reaction involved in dissolving the copper oxide in ferric sulfate and the well-known Marguerite reaction of the titration are as follows:

$$Cu_2O + Fe_2(SO_4)_3 + H_2SO_4 \rightarrow 2CuSO_4 + 2FeSO_4 + H_2O$$

$$10FeSO_4 + 8H_2SO_4 + 2KMnO_4 \rightarrow 5Fe_2(SO_4)_3 + K_2SO_4 + 2MnSO_4 + 8H_2O$$

REAGENTS. *Copper Sulfate Solution.* Dissolve 40 g. of $CuSO_4 \cdot 5H_2O$ in water and make up to 1 liter.

Alkaline Rochelle Salt Solution. Dissolve 200 g. of $KNaC_4H_4O_6 \cdot 4H_2O$ and 150 g. of NaOH in water and make up to 1 liter.

Acid Ferric Sulfate Solution. Dissolve 50 g. of $Fe(SO_4)_3$ in water, add 200 g. of H_2SO_4, and dilute to 1 liter.

Standard Potassium Permanganate Solution. Dissolve 5 g. of crystalline $KMnO_4$ in water and make up to exactly 1 liter. For approximate calculations 1 ml. of this solution may be regarded as equivalent to 1 mg. of metallic copper, but for exact work the solution should be standardized against sodium oxalate or arsenic trioxide, or preferably by conducting a series of actual determinations on one or more of the pure sugars given in Bertrand's table, weighing in each case first the cuprous oxide, then dissolving in $Fe(SO_4)_3$ and titrating with the permanganate solution as directed below.

Bertrand used ammonium oxalate,

$$(NH_4)_2C_2O_4 \cdot H_2O = 142.1,$$

and multiplied the weight of oxalate by $(63.6 \times 2)/142.1 = 0.8951$.

PROCESS. *Copper Reduction and Filtration.* Pipet into a 125-ml. Erlenmeyer flask 20 ml. of the solution or extract of the sample, containing 10 to 90 mg. of reducing sugar, and add 20 ml. each of the *copper sulfate* and *alkaline Rochelle salt solutions.* Heat the mixture to boiling, continue the boiling for exactly 3 minutes, then remove the heat. After allowing the precipitate of cuprous oxide to settle for a few seconds, decant as rapidly as possible on a Gooch crucible or other form of asbestos filter that completely retains the precipitate. Wash in such a manner as to leave as much as possible of the cuprous oxide in the flask. If the color of the liquid does not show an excess of copper, start the determination afresh.

Ferric Sulfate Treatment. Pass small portions (5 to 20 ml.) of *acid ferric sulfate solution* through the filter into the precipitation flask, then if the least trace of the precipitate has not dissolved to a sea-green solution, return the liquid to the filter, repeating the operation with addition, if necessary, of small fresh portions of the solvent sufficient for complete solution. Finally wash the filter with water, collecting the washings in the precipitation flask which serves also for the titration.

Titration. Add the *standard permanganate solution* to the ferric sulfate solution until a single drop changes the color from green to a light rose tint and a second drop produces

Reducing Sugars from Copper (Bertrand)

Mg. of Sugar	Mg. of Copper Equivalent to				
	Invert sugar	Dex-trose	Galac-tose	Mal-tose	Lac-tose
10	20.6	20.4	19.3	11.2	14.4
11	22.6	22.4	21.2	12.3	15.8
12	24.6	24.3	23.0	13.4	17.2
13	26.5	26.3	24.9	14.5	18.6
14	28.5	28.3	26.7	15.6	20.0
15	30.5	30.2	28.6	16.7	21.4
16	32.5	32.2	30.5	17.8	22.8
17	34.5	34.2	32.3	18.9	24.2
18	36.4	36.2	34.2	20.0	25.6
19	38.4	38.1	36.0	21.1	27.0
20	40.4	40.1	37.9	22.2	28.4
21	42.3	42.0	39.7	23.3	29.8
22	44.2	43.9	41.6	24.4	31.1
23	46.1	45.8	43.4	25.5	32.5
24	48.0	47.7	45.2	26.6	33.9
25	49.8	49.6	47.0	27.7	35.2
26	51.7	51.5	48.9	28.9	36.6
27	53.6	53.4	50.7	30.0	38.0
28	55.5	55.3	52.5	31.1	39.4
29	57.4	57.2	54.4	32.2	40.7
30	59.3	59.1	56.2	33.3	42.1
31	61.1	60.9	58.0	34.4	43.4
32	63.0	62.8	59.7	35.5	44.8
33	64.8	64.6	61.5	36.5	46.1
34	66.7	66.5	63.3	37.6	47.4
35	68.5	68.3	65.0	38.7	48.7
36	70.3	70.1	66.8	39.8	50.1
37	72.2	72.0	68.6	40.9	51.4
38	74.0	73.8	70.4	41.9	52.7
39	75.9	75.7	72.1	43.0	54.1
40	77.7	77.5	73.9	44.1	55.4
41	79.5	79.3	75.6	45.2	56.7
42	81.2	81.1	77.4	46.3	58.0
43	83.0	82.9	79.1	47.4	59.3
44	84.8	84.7	80.8	48.5	60.6
45	86.5	86.4	82.5	49.5	61.9
46	88.3	88.2	84.3	50.6	63.3
47	90.1	90.0	86.0	51.7	64.6
48	91.9	91.8	87.7	52.8	65.9
49	93.6	93.6	89.5	53.9	67.2
50	95.4	95.4	91.2	55.0	68.5
51	97.1	97.1	92.9	56.1	69.8
52	98.8	98.9	94.6	57.1	71.1
53	100.6	100.6	96.3	58.2	72.4
54	102.2	102.3	98.0	59.3	73.7
55	104.0	104.1	99.7	60.3	74.9
56	105.7	105.8	101.5	61.4	76.2
57	107.4	107.6	103.2	62.5	77.5
58	109.2	109.3	104.9	63.5	78.8
59	110.9	111.1	106.6	64.6	80.1
60	112.6	112.8	108.3	65.7	81.4
61	114.3	114.5	110.0	66.8	82.7
62	115.9	116.2	111.6	67.9	83.9
63	117.6	117.9	113.3	68.9	85.2
64	119.2	119.6	115.0	70.0	86.5
65	120.9	121.3	116.6	71.1	87.7
66	122.6	123.0	118.3	72.2	89.9
67	124.2	124.7	120.0	73.3	90.3
68	125.9	126.4	121.7	74.3	91.6
69	127.5	128.1	123.3	75.4	92.8
70	129.2	129.8	125.0	76.5	94.1
71	130.8	131.4	126.6	77.6	95.4
72	132.4	133.1	128.3	78.6	96.9
73	134.0	134.7	130.0	79.7	98.0
74	135.6	136.3	131.5	80.8	99.1
75	137.2	137.9	133.1	81.8	100.4
76	138.9	139.6	134.8	82.9	101.7
77	140.5	141.2	136.4	84.0	102.9
78	142.1	142.8	138.0	85.1	104.2
79	143.7	144.5	139.7	86.1	105.4
80	145.3	146.1	141.3	87.2	106.7
81	146.9	147.7	142.9	88.3	107.9
82	148.5	149.3	144.6	89.4	109.2
83	150.0	150.9	146.2	90.4	110.4
84	151.6	152.5	147.8	91.5	111.7
85	153.2	154.0	149.4	92.6	112.9
86	154.8	155.6	151.1	93.7	114.1
87	156.4	157.2	152.7	94.8	115.4
88	157.9	158.8	154.3	95.8	116.6
89	159.5	160.4	156.0	96.9	117.9
90	161.1	162.0	157.6	98.0	119.1
91	162.6	163.6	159.2	99.0	120.3
92	164.2	165.2	160.8	100.1	121.6
93	165.7	166.7	162.4	101.1	122.8
94	167.3	168.3	164.0	102.2	124.0
95	168.8	169.9	165.6	103.2	125.2
96	170.3	171.5	167.2	104.2	126.5
97	171.9	173.1	168.8	105.3	127.7
98	173.4	174.6	170.4	106.3	128.9
99	175.0	176.2	172.0	107.4	130.2
100	176.5	177.8	173.6	108.4	131.4

an intense rose coloration. The titration may be carried out in artificial light.

CALCULATION. In the first equation above, 1 atom of copper is equivalent to 1 atom of iron and in the second equation 5 atoms of iron are equivalent to 1 molecule of potassium permanganate; 1 ml. of the standard potassium permanganate solution contains 5 mg. of $KMnO_4$ which is equivalent to $(63.57 \times 5)/[2(158.03)] = 1.0055$ mg. of copper. To obtain the number of milligrams of copper, multiply the number of milliliters of standard potassium permanganate solution required for the titration by 1.0055; to obtain the corresponding weight of sugar consult Bertrand's table above.

I. Canals Modification.[34] Seeking to make the conditions accurately reproducible, Canals conducts the reduction with 40 ml. (20 + 20) of the *alkaline copper solution* in a glass tube 7.5 cm. in diameter, immersed in a boiling water bath for 10 minutes, then allows to cool 2 minutes before filtering.

II. Sichert and Bleyer Modification for Dextrose, Maltose, and Dextrin.[35] This procedure originated at Munich University.

REAGENTS. *Fehling Reagent.* (1) *Copper Sulfate Solution.* Dissolve 69.28 g. of $CuSO_4 \cdot 5H_2O$ in water and make up to 1 liter; (2) *Alkaline Tartrate Solution.* Dissolve 346 g. of $KNaC_4H_4O_6 \cdot 4H_2O$ and 106 g. of NaOH in water and make up to 1 liter.

Sodium Acetate Solution, 5%.

Acid Ferric Alum Solution. Make 120 g. of $FeNH_4(SO_4)_2 \cdot 12H_2O$ and 100 ml. of H_2SO_4 up to 1 liter with water.

Standard Potassium Permanganate Solution, 0.1 *N.*

PROCESS. A. *Dextrose.* Place in a 200-ml. Erlenmeyer flask 10 ml. of *copper sulfate solution,* 20 ml. of *5% sodium acetate solution,* 10 ml. of the solution containing 100 mg. of the sugar, and 10 ml. of water (20 ml. of the sugar solution and no water for small amounts of dextrose). Heat in a boiling water bath for 20 minutes, collect the copper oxide in a Gooch crucible or filtering tube, and wash three times with hot water. Dissolve the precipitate in 20 ml. of *ferric alum solution* and after 3 minutes titrate with *standard 0.1 N potassium permanganate solution.*

In the Table for Process A find the weight of dextrose corresponding to the number of milliliters of potassium permanganate required for the titration and calculate *a*, the weight of dextrose per 100 g. of the sample.

B. *Dextrose plus Maltose.* Mix in a 300-ml. Erlenmeyer flask 25 ml. each of *copper sulfate solution, alkaline Rochelle salt solution,* and the sugar solution (if rich in dextrose, use 10 ml. of the sugar solution and 15 ml. of water), heat 20 minutes in a boiling water bath, filter, dissolve in *acid ferric alum solution,* and titrate as above, but without waiting 3 minutes.

Find in the Table for Processes B and C the weight of sugars expressed as dextrose corresponding to the number of milliliters of potassium permanganate required for the titration and calculate *b*, the weight of sugars per 100 g. of the sample.

C. *Total Carbohydrates* (Dextrose, Maltose, and Dextrin). Heat 200 ml. of the sugar solution in a 500-ml. volumetric flask for 3 hours in a boiling water bath with 20 ml. of *25% hydrochloric acid,* cool, neutralize with *strong sodium hydroxide solution,* and make up to 500 ml. Add to a 25-ml. aliquot of the hydrolyzed solution a mixture of 25 ml. each of *copper sulfate solution* and *alkaline Rochelle salt solution* and proceed as under B.

Find in the Table for processes B and C the weight of sugars expressed as dextrose corresponding to the number of milliliters of potassium permanganate required for the titration and calculate *c*, the weight of sugars per 100 g. of the sample.

CALCULATION. Obtain the percentages of the three carbohydrates as follows: dextrose $= a$; maltose $= 2(b - a)$; dextrin $= 0.9(a + c - 2b)$.

Calculation of Dextrose by Process A (Sichert and Bleyer)

0.1 N $KMnO_4$	Mg. of Dextrose									
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
ml.										
10	26.5	26.8	27.1	27.4	27.8	28.1	28.4	28.7	29.0	29.3
11	29.7	30.0	30.4	30.7	31.1	31.5	31.8	32.2	32.6	32.9
12	33.3	33.7	34.1	34.5	34.9	35.4	35.8	36.2	36.6	37.0
13	37.4	37.9	38.4	38.8	39.3	39.8	40.3	40.7	41.2	41.7
14	42.2	42.7	43.2	43.8	44.3	44.9	45.4	46.0	46.5	47.0
15	47.6	48.2	48.8	49.4	50.1	50.7	51.3	51.9	52.5	53.2
16	53.8	54.5	55.2	55.9	56.6	57.3	58.0	58.7	59.4	60.2
17	60.9	61.7	62.5	63.3	64.1	64.9	65.7	66.5	67.4	68.2
18	69.0	69.9	70.9	71.9	72.8	73.8	74.8	75.7	76.7	77.6
19	78.6	79.6	80.7	81.7	82.7	83.7	84.8	85.8	86.8	87.8
20	88.9	90.0	91.2	92.3	93.5	94.7	96.0	97.2	98.5	99.7

Calculation of Sugars by Processes B and C (Sichert and Bleyer)

0.1 N $KMnO_4$	Sugars	0.1 N $KMnO_4$	Sugars	0.1 N $KMnO_4$	Sugars	Interpolations	
ml.	mg.	ml.	mg.	ml.	mg.		
10	30.64	20	62.89	30	95.14	0.05	0.16
11	33.86	21	66.11	31	98.36	0.1	0.32
12	37.09	22	69.34	32	101.59	0.2	0.65
13	40.31	23	72.56	33	104.81	0.3	0.97
14	43.54	24	75.79	34	108.04	0.4	1.29
15	46.76	25	79.02	35	111.27	0.5	1.61
16	49.99	26	82.24	36	114.49	0.6	1.94
17	53.21	27	85.47	37	117.71	0.7	2.26
18	56.44	28	88.69	38	120.94	0.8	2.58
19	59.66	29	91.91	39	124.16	0.9	2.90

III. Tul'chinskiĭ Glycerol Modification.[36] The novel feature of this modification is the substitution of glycerol for Rochelle salt. The solutions employed are (1) 20 ml. of *4% crystalline copper sulfate solution*, (2) 5 ml. of *glycerol solution* (212 g. per liter), (3) 15 ml. of *20% potassium hydroxide solution*, and (4) 20 ml. of the solution of the sample. In order to compensate for the difference in the reducing power of the Bertrand and Tul'chinskiĭ solutions and make the Bertrand table applicable, a calculation is necessary in accordance with the following equation:

$$B = G \pm b \pm d$$

in which B is the weight of copper for use in

the Bertrand table corresponding to G, the weight obtained by the glycerol modification, b is the difference in the blanks by the Rochelle salt and the glycerol procedures, and d is a correction for the error due to the concentration which for 100 mg. of dextrose is 1, but for higher concentrations may reach 4%.

IV. Klemen and Skerlak Modification.[37] These authors use a small flask for the copper reduction to avoid oxidation through contact with the air. They confirm Josephson's conclusion that the method applied to maltose gives slightly higher results than the Willstätter and Schudel modification of the Romijn method but is sufficiently accurate for most purposes.

V. Phillips Semi-Micro Modification.[38] This simple modification (New Hampshire Experiment Station) deserves careful study.

PROCESS. *Copper Reduction.* To 10 ml. of the sugar solution, add 5 ml. of *20% cupric sulfate solution* and 5 ml. of a solution containing 50 g. of *Rochelle salt*, 40 g. of *sodium bicarbonate*, and 50 g. of *anhydrous sodium carbonate* per liter. Heat in a boiling water bath for 15 minutes, collect the precipitate of copper suboxide on a Gooch crucible, and wash with water.

Titration. Dissolve the precipitate in 10 ml. of *10% neutral ferric alum solution*, wash with 3 ml. of *18 N sulfuric acid*, and then wash with water. Add to the solution 3 ml. of *25% phosphoric acid* and a drop of *0.025 M o-phenanthroline ferrous sulfate complex indicator*. Titrate the ferrous iron with *standard 0.01 N potassium permanganate solution*.

Correct for the permanganate consumed in a blank determination.

CALCULATION. Obtain the milligrams of dextrose (D), levulose (L), and sucrose (S) from the milliliters of permanganate solution (P) by the following formulas: $D = 0.018 + 0.212P$; $L = 0.026 + 0.221P$; $S = 0.022 + 0.205P$.

Lane and Eynon Unified Copper Reduction Volumetric Method.[39] This method, as explained below, takes cognizance in the calculation tables of the difference in reducing factors of solutions of the common sugars (dextrose, levulose, invert sugar, maltose, and lactose) at different concentrations, alone and in the presence of different amounts of non-reducing sugars. By the use of methylene blue indicator, a sharp end-point is obtained.

APPARATUS. *Special Buret.* The delivery tube is double-bent so that the sugar solution is not delivered directly into the boiling copper solution. It is provided with a ball- or pinchcock, not a glass cock that is affected by heat. The same stand suffices for supporting the buret by a clamp and the flask over wire gauze on a ring where it remains during the whole titration.

REAGENTS. *Fehling-Soxhlet Solutions 1 and 2* (see above).

Methylene Blue Indicator, 1% aqueous solution.

Pure Sugars. Lane and Eynon describe the preparation of the pure sugars, which are of value if the analyst seeks to check the tables or correct for personal equation. Pure sugars are also supplied by the U. S. National Bureau of Standards.

PROCESS. *Preliminary Titration.* Prepare a solution of the sample of such concentration that the weight of the sugar per 100 ml. falls within the limits of the table.

Pipet 5 or 12.5 ml. each of *Fehling-Soxhlet solutions 1 and 2* into a 300- to 400-ml. flask, add 15 ml. of the sugar solution from a buret, and without further dilution heat to boiling over wire gauze. If after about 15 seconds of boiling the copper appears to be almost all reduced, as indicated by the bright red suspended cuprous oxide, add a few drops of *1% methylene blue indicator*, continue the boiling 1 to 2 minutes longer, then add successive amounts of 1 ml. (or less) of the sugar solution, boiling 10 seconds between the additions, until the color is completely discharged. If, however, after the first addition

CALCULATION TABLE FOR 10 AND 25 ML. OF FEHLING-SOXHLET SOLUTION (LANE AND EYNON)
(Weights in milligrams of invert sugar per 100 ml. of solution)

Sugar Sol.	Invert Sugar (Sucrose 0 g.)	Invert Sugar (Sucrose 0 g.)	Invert Sugar (Sucrose 1 g.)	Invert Sugar (Sucrose 1 g.)	Invert Sugar (Sucrose 5 g.)	Invert Sugar (Sucrose 10 g.)	Invert Sugar (Sucrose 25 g.)
ml.	10 ml.	25 ml.	10 ml.	25 ml.	10 ml.	10 ml.	10 ml.
15	336.	824.	333.	817.	317.	307.	289
16	316.	772.	312.	767.	297.	288.	271
17	298.	727.	295.	721.	280.	271.	255
18	282.	687.	278.	682.	264.	256.	240
19	267.	651.	264.	646.	250.	243.	227
20	254.5	619.0	251.0	614.0	238.0	230.5	216
21	242.9	589.5	239.0	584.8	226.7	219.5	206
22	231.8	563.2	228.2	558.2	216.4	209.5	196
23	222.2	538.7	218.7	534.0	207.0	200.4	187
24	213.3	516.7	209.8	512.1	198.3	192.1	179
25	204.8	496.0	201.6	492.0	190.4	184.0	171
26	197.4	477.3	193.8	473.1	183.1	176.9	164
27	190.4	459.7	186.7	455.6	176.4	170.4	158
28	183.7	443.6	180.2	439.6	170.3	164.3	152
29	177.6	428.3	174.1	424.4	164.5	158.6	147
30	171.7	414.3	168.3	410.4	159.0	153.3	142
31	166.3	401.0	163.1	397.4	153.9	148.1	137
32	161.2	388.7	158.1	385.0	149.1	143.4	132
33	156.6	377.0	153.3	373.4	144.5	139.1	128
34	152.2	366.2	148.9	362.6	140.3	134.9	124
35	147.9	355.8	144.7	352.3	136.3	130.9	121
36	143.9	346.1	140.7	342.5	132.5	127.1	117
37	140.2	336.8	137.0	333.5	128.9	123.5	114
38	136.6	328.1	133.5	324.7	125.5	120.3	111
39	133.3	319.7	130.2	316.4	122.3	117.1	107
40	130.1	311.9	127.0	308.6	119.2	114.1	104
41	127.1	304.4	123.9	301.2	116.3	111.2	102
42	124.2	297.3	121.0	294.1	113.5	108.5	99
43	121.4	290.5	118.2	287.3	110.9	105.8	97
44	118.7	284.1	115.6	280.9	108.4	103.4	94
45	116.1	277.9	113.1	274.7	106.0	101.0	92
46	113.7	272.0	110.6	268.7	103.7	98.7	90
47	111.4	266.3	108.2	263.1	101.5	96.4	88
48	109.2	260.8	106.0	257.7	99.4	94.3	86
49	107.1	255.5	104.0	252.5	97.4	92.3	84
50	105.1	250.6	102.0	247.6	95.4	90.4	82

CALCULATION TABLE FOR 10 AND 25 ML. OF FEHLING-SOXHLET SOLUTION (LANE AND EYNON)—*Concluded*

(Weights in milligrams of individual reducing sugars per 100 ml. of solution)

Sugar Sol.	Dextrose		Levulose		Anhydrous Maltose		Hydrated Maltose *		Anhydrous Lactose		Hydrated Lactose *	
ml.	10 ml.	25 ml.	10 ml.	25 ml.	10 ml.	25 ml.	10 ml.	25 ml.	10 ml.	25 ml.	10 ml.	25 ml.
15	327.	801.	348.	849.	515.	1319.	542.	1388.	432.	1093.	455.	1150.
16	307.	751.	327.	796.	482.	1233.	507.	1298.	405.	1022.	426.	1076.
17	289.	707.	308.	750.	453.	1159.	477.	1220.	381.	960.	401.	1010.
18	274.	668.	291.	708.	427.	1093.	450.	1151.	359.	906.	378.	952.
19	260.	633.	276.	672.	405.	1034.	426.	1088.	340.	855.	358.	900.
20	247.4	601.5	262.5	638.0	383.8	980.7	404.0	1032.3	323.0	811.8	340.0	854.5
21	235.8	572.9	250.6	608.1	365.1	932.5	384.3	981.6	307.6	772.3	323.8	812.4
22	225.5	547.3	239.6	580.6	348.1	888.7	366.4	935.5	293.6	735.8	309.1	774.5
23	216.1	523.6	229.1	555.5	332.5	848.5	350.0	893.2	280.6	703.0	295.4	740.0
24	207.4	501.9	220.0	532.5	318.3	811.8	335.0	854.5	268.8	673.1	282.9	708.5
25	199.3	482.0	211.3	511.5	305.4	778.1	321.5	819.0	258.0	645.5	271.6	679.5
26	191.8	463.7	203.3	491.9	293.4	747.0	308.8	786.3	248.0	620.1	261.0	652.7
27	184.9	446.8	196.0	474.0	282.2	718.2	297.0	756.0	238.5	596.5	251.1	627.9
28	178.5	431.1	189.3	457.2	271.8	691.5	286.1	727.9	230.0	574.6	242.1	604.8
29	172.5	416.4	183.1	441.6	262.2	666.6	276.0	701.7	222.2	554.1	233.8	583.3
30	167.0	402.7	177.2	427.0	253.3	643.4	266.6	677.3	214.7	535.1	226.0	563.3
31	161.8	389.7	171.7	413.3	244.9	621.6	257.8	654.3	207.8	517.6	218.7	544.8
32	156.9	377.6	166.5	400.5	237.2	601.4	249.7	633.1	201.3	501.0	211.9	527.4
33	152.4	366.3	161.6	388.5	229.8	582.4	241.9	613.0	195.3	485.5	205.6	511.0
34	148.0	355.6	157.0	377.3	222.9	564.6	234.6	594.3	189.7	470.8	199.7	495.6
35	143.9	345.6	152.6	366.7	216.2	547.7	227.6	576.5	184.3	457.0	194.0	481.1
36	140.0	336.3	148.6	356.6	210.0	531.7	221.1	559.7	179.2	443.9	188.6	467.3
37	136.4	327.4	144.7	347.0	204.3	516.7	215.0	543.9	174.3	431.6	183.5	454.3
38	132.9	318.8	140.9	338.1	198.7	502.5	209.2	528.9	169.8	420.0	178.7	442.1
39	129.6	310.7	137.3	329.6	193.6	489.0	203.8	514.7	165.4	409.0	174.1	430.5
40	126.5	303.1	134.0	321.5	188.6	476.2	198.5	501.3	161.2	398.5	169.7	419.5
41	123.6	295.9	130.9	313.7	184.3	464.1	193.7	488.5	157.6	388.6	165.9	409.0
42	120.8	289.0	127.9	306.2	179.4	452.5	188.8	476.3	153.8	379.1	161.9	399.1
43	118.1	282.4	125.1	299.2	175.1	441.5	134.3	464.7	150.2	370.2	158.1	389.7
44	115.5	276.1	122.4	292.5	171.0	430.9	180.0	453.6	147.0	361.7	154.7	380.7
45	113.0	270.1	119.8	286.2	167.1	420.9	175.9	443.0	143.7	353.5	151.3	372.1
46	110.6	264.3	117.2	280.0	163.4	411.4	172.0	433.1	140.6	345.7	148.0	363.9
47	108.4	258.8	114.7	274.2	159.9	402.4	168.3	423.6	137.8	338.2	145.1	356.0
48	106.2	253.5	112.4	268.6	156.5	393.7	164.7	414.4	135.0	330.9	142.1	348.3
49	104.1	248.4	110.2	263.2	153.1	385.2	161.2	405.5	132.2	324.0	139.2	341.0
50	102.2	243.6	108.0	258.0	150.1	377.3	158.0	397.2	129.8	317.5	136.6	334.2

* $C_{12}H_{22}O_{11} \cdot H_2O$.

of 15 ml. of sugar solution and 15 seconds of boiling a large amount of copper remains unreduced, add 10 ml. of the sugar solution and boil for 15 seconds, repeating the addition and boiling until the end-point appears near, then add the indicator, boil 1 to 2 minutes, and finish the titration with small additions as at the start.

Final Titration. To the *Fehling-Soxhlet solutions 1 and 2* in the flask, measured as in the preliminary titration, add if possible within 1 ml. of the required amount of the sugar solution. Heat over gauze to boiling and continue to boil gently 2 minutes, then while still boiling add 3 to 5 drops of *methylene blue indicator* and complete the titration, continuing the boiling to a total of 3 minutes without interruption.

CALCULATION. Obtain from the tables herewith the milligrams of the sugar per 100 ml. of the solution of the sample corresponding to the milliliters of sugar solution used in the titration. The two tables are a consolidation of six given by Lane and Eynon, but the so-called factor figures, which seem superfluous for the present purpose, are omitted. If desired, these latter may be derived as noted under the Jackson Modification below.

A study of the calculation tables will show that the reduction is not strictly in proportion to the amount of sugar, but is influenced by the concentration. For example, the factor for a solution of invert sugar, 50 ml. of which reduce the copper in 10 ml. of Fehling-Soxhlet solution, is about 4% higher than that of a solution of which 15 ml. reduce the same volume of solution and amount of copper, whereas in the presence of 25 g. of sucrose per 100 ml. of the invert sugar solution it is approximately 5% lower.

Jackson Modification.[40] The salient feature of this procedure (U. S. National Bureau of Standards), tentatively adopted by the A.O.A.C., is the comparison of the data on the pure sugars as obtained by each analyst with those obtained on the unknown by exactly the same technique.

PROCESS. *Titration of Standard Solution.* Prepare a standard solution of the pure sugar of such a concentration that 15 to 50 ml. will reduce all the copper. Obtain the approximate titer by dividing the factor by the number of milligrams of sugar in 1 ml., then proceed as directed for the final titration in the original method above. Multiply the titer by the number of milligrams in 1 ml. of the standard solution and compare the factor thus obtained with the calculated or tabulated factor to determine the correction, if any. If results within 1% are sufficient for the purpose, standardization may be omitted.

Preliminary Titration of the Unknown. If the approximate sugar concentration of the sample is not known, add 15 ml. of the sugar solution to 5 or 12.5 ml. each of *Fehling-Soxhlet solutions 1 and 2,* heat to boiling over wire gauze, and boil 15 seconds. Run in rapidly additional amounts of the sugar solution until only a faint blue color remains, then add 2 to 5 drops of *methylene blue* and complete the titration.

Final Titration of the Unknown. Proceed as in the final titration of the Lane and Eynon method.

Factors. For an occasional determination, obtain the factor by multiplying the number of milligrams of sugar per 10 or 25 ml. (second or third column of the Lane and Eynon table) by the corresponding number of milliliters of the sugar solution (first column) and dividing the product by 100. For routine work, the tables in *Methods of Analysis,* A.O.A.C., are convenient.

CALCULATION. Obtain the milligrams of sugar in 100 ml. (M) by the formula

$$M = \frac{F \times 100}{T}$$

in which F is the factor and T is the titer.

REDUCING SUGARS FROM CUPROUS OXIDE AND COPPER (MUNSON AND WALKER-HAMMOND-GIVEN) *

Cu_2O	Cu	Dextrose	Levulose †	Invert Sugar	Invert Sugar in Presence of Sucrose		Lactose $C_{12}H_{22}O_{11}\cdot H_2O$‡	Lactose in Presence of Sucrose ‡		Maltose $C_{12}H_{22}O_{11}\cdot H_2O$
					0.4 g. total sugar	2 g. total sugar		1 : 4	1 : 12	
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
10	8.9	4.0	4.5	4.5	1.6	....	6.3	6.1	....	6.2
12	10.7	4.9	5.5	5.4	2.5	....	7.5	7.3	....	7.9
14	12.4	5.7	6.4	6.3	3.4	....	8.8	8.5	....	9.5
16	14.2	6.6	7.3	7.2	4.3	....	10.0	9.7	....	11.2
18	16.0	7.5	8.3	8.1	5.2	....	11.3	10.9	....	12.9
20	17.8	8.3	9.2	8.9	6.1	....	12.5	12.1	....	14.6
22	19.5	9.2	10.2	9.8	7.0	....	13.8	13.3	....	16.2
24	21.3	10.0	11.1	10.7	7.9	....	15.0	14.5	....	17.9
26	23.1	10.9	12.1	11.6	8.8	....	16.3	15.8	....	19.6
28	24.9	11.8	13.0	12.5	9.7	....	17.6	17.0	....	21.2
30	26.6	12.6	14.0	13.4	10.7	4.3	18.8	18.2	....	22.9
32	28.4	13.5	14.9	14.3	11.6	5.2	20.1	19.4	....	24.6
34	30.2	14.3	15.9	15.2	12.5	6.1	21.4	20.7	....	26.2
36	32.0	15.2	16.8	16.1	13.4	7.0	22.8	22.0	....	27.9
38	33.8	16.1	17.8	16.9	14.3	7.9	24.2	23.3	....	29.6
40	35.5	16.9	18.7	17.8	15.2	8.8	25.5	24.7	....	31.3
42	37.3	17.8	19.7	18.7	16.1	9.7	26.9	26.0	....	32.9
44	39.1	18.7	20.6	19.6	17.0	10.7	28.3	27.3	....	34.6
46	40.9	19.6	21.6	20.5	17.9	11.6	29.6	28.6	....	36.3
48	42.6	20.4	22.6	21.4	18.8	12.5	31.0	30.0	....	37.9
50	44.4	21.3	23.5	22.3	19.7	13.4	32.3	31.3	....	39.6
52	46.2	22.2	24.5	23.2	20.7	14.3	33.7	32.6	....	41.3
54	48.0	23.0	25.4	24.1	21.6	15.2	35.1	34.0	....	42.9
56	49.7	23.9	26.4	25.0	22.5	16.2	36.4	35.3	....	44.6
58	51.5	24.8	27.4	25.9	23.4	17.1	37.8	36.6	....	46.3
60	53.3	25.6	28.3	26.8	24.3	18.0	39.2	37.9	....	48.0
62	55.1	26.5	29.3	27.7	25.2	18.9	40.5	39.3	....	49.6
64	56.8	27.4	30.2	28.6	26.2	19.8	41.9	40.6	....	51.3
66	58.6	28.3	31.2	29.5	27.1	20.8	43.3	41.9	....	53.0
68	60.4	29.2	32.2	30.4	28.0	21.7	44.7	43.3	40.7	54.6
70	62.2	30.0	33.1	31.3	28.9	22.6	46.0	44.6	41.9	56.3
72	64.0	30.9	34.1	32.3	29.8	23.5	47.4	45.9	43.1	58.0
74	65.7	31.8	35.1	33.2	30.8	24.5	48.8	47.3	44.2	59.6
76	67.5	32.7	36.0	34.1	31.7	25.4	50.1	48.6	45.4	61.3
78	69.3	33.6	37.0	35.0	32.6	26.3	51.5	49.9	46.6	63.0
80	71.1	34.4	38.0	35.9	33.5	27.3	52.9	51.3	47.8	64.6
82	72.8	35.3	38.9	36.8	34.5	28.2	54.2	52.6	49.0	66.3
84	74.6	36.2	39.9	37.7	35.4	29.1	55.6	53.9	50.1	68.0
86	76.4	37.1	40.9	38.6	36.3	30.0	57.0	55.3	51.3	69.7
88	78.2	38.0	41.8	39.5	37.2	31.0	58.4	56.6	52.5	71.3

* *U. S. Bur. Stand. Circ.* **44,** p. 139; *Methods of Analysis of the A.O.A.C.*
† Hammond: *J. Research Natl. Bur. Standards* 1940, **24,** 579, RP1301.
‡ Given: *Methods of Sugar Analysis and Allied Determinations.*

Reducing Sugars from Cuprous Oxide and Copper (Munson and Walker-Hammond-Given)—*Continued*

Cu_2O	Cu	Dextrose	Levulose	Invert Sugar	Invert Sugar in Presence of Sucrose		Lactose $C_{12}H_{22}O_{11} \cdot H_2O$	Lactose in Presence of Sucrose		Maltose $C_{12}H_{22}O_{11} \cdot H_2O$
					0.4 g. total sugar	2 g. total sugar		1 : 4	1 : 12	
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
90	79.9	38.9	42.8	40.4	38.2	31.9	59.7	57.9	53.7	73.0
92	81.7	39.8	43.8	41.4	39.1	32.8	61.1	59.3	54.9	74.7
94	83.5	40.6	44.8	42.3	40.0	33.8	62.5	60.6	56.0	76.3
96	85.3	41.5	45.7	43.2	41.0	34.7	63.8	61.9	57.2	78.0
98	87.1	42.4	46.7	44.1	41.9	35.6	65.2	63.3	58.4	79.7
100	88.8	43.3	47.7	45.0	42.8	36.6	66.6	64.6	59.6	81.3
102	90.6	44.2	48.6	46.0	43.8	37.5	68.0	66.0	60.8	83.0
104	92.4	45.1	49.6	46.9	44.7	38.5	69.3	67.3	62.0	84.7
106	94.2	46.0	50.6	47.8	45.6	39.4	70.7	68.6	63.2	86.3
108	95.9	46.9	51.6	48.7	46.6	40.3	72.1	70.0	64.4	88.0
110	97.7	47.8	52.6	49.6	47.5	41.3	73.5	71.3	65.6	89.7
112	99.5	48.7	53.5	50.6	48.4	42.2	74.8	72.6	66.7	91.3
114	101.3	49.6	54.5	51.5	49.4	43.2	76.2	74.0	67.9	93.0
116	103.0	50.5	55.5	52.4	50.3	44.1	77.6	75.3	69.1	94.7
118	104.8	51.4	56.5	53.3	51.2	45.0	79.0	76.7	70.3	96.4
120	106.6	52.3	57.5	54.3	52.2	46.0	80.3	78.0	71.5	98.0
122	108.4	53.2	58.4	55.2	53.1	46.9	81.7	79.3	72.7	99.7
124	110.1	54.1	59.4	56.1	54.1	47.9	83.1	80.7	73.9	101.4
126	111.9	55.0	60.4	57.0	55.0	48.8	84.5	82.0	75.1	103.0
128	113.7	55.9	61.4	58.0	55.9	49.8	85.8	83.4	76.3	104.7
130	115.5	56.8	62.4	58.9	56.9	50.7	87.2	84.7	77.5	106.4
132	117.3	57.7	63.4	59.8	57.8	51.7	88.6	86.0	78.7	108.0
134	119.0	58.6	64.3	60.8	58.8	52.6	90.0	87.4	79.7	109.7
136	120.8	59.5	65.3	61.7	59.7	53.6	91.3	88.7	81.1	111.4
138	122.6	60.4	66.3	62.6	60.7	54.5	92.7	90.1	82.3	113.0
140	124.4	61.3	67.3	63.6	61.6	55.5	94.1	91.4	83.5	114.7
142	126.1	62.2	68.3	64.5	62.6	56.4	95.5	92.8	84.7	116.4
144	127.9	63.1	69.3	65.4	63.5	57.4	96.8	94.1	85.9	118.0
146	129.7	64.0	70.3	66.4	64.5	58.3	98.2	95.4	87.1	119.7
148	131.5	65.0	71.3	67.3	65.4	59.3	99.6	96.8	88.3	121.4
150	133.2	65.9	72.2	68.3	66.4	60.2	101.0	98.1	89.5	123.0
152	135.0	66.8	73.2	69.2	67.3	61.2	102.3	99.5	90.8	124.7
154	136.8	67.7	74.2	70.1	68.3	62.1	103.7	100.8	92.0	126.4
156	138.6	68.6	75.2	71.1	69.2	63.1	105.1	102.2	93.2	128.0
158	140.3	69.5	76.2	72.0	70.2	64.1	106.5	103.5	94.4	129.7
160	142.1	70.4	77.2	73.0	71.2	65.0	107.9	104.8	95.6	131.4
162	143.9	71.4	78.2	73.9	72.1	66.0	109.2	106.2	96.8	133.0
164	145.7	72.3	79.2	74.9	73.1	66.9	110.6	107.5	98.0	134.7
166	147.5	73.2	80.2	75.8	74.0	67.9	112.0	108.9	99.2	136.4
168	149.2	74.1	81.2	76.8	75.0	68.9	113.4	110.2	100.4	138.0
170	151.0	75.1	82.2	77.7	76.0	69.8	114.8	111.6	101.6	139.7
172	152.8	76.0	83.2	78.7	76.9	70.8	116.1	112.9	102.8	141.4
174	154.6	76.9	84.2	79.6	77.9	71.7	117.5	114.3	104.1	143.0
176	156.3	77.8	85.2	80.6	78.8	72.7	118.9	115.6	105.3	144.7
178	158.1	78.8	86.2	81.5	79.8	73.7	120.3	117.0	106.5	146.4

SUGARS FROM CUPROUS OXIDE AND COPPER (MUNSON AND WALKER-HAMMOND-GIVEN)—*Continued*

Cu_2O	Cu	Dextrose	Levulose	Invert Sugar	Invert Sugar in Presence of Sucrose		Lactose $C_{12}H_{22}O_{11} \cdot H_2O$	Lactose in Presence of Sucrose		Maltose $C_{12}H_{22}O_{11} \cdot H_2O$
					0.4 g. total sugar	2 g. total sugar		1 : 4	1 : 12	
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
180	159.9	79.7	87.2	82.5	80.8	74.6	121.6	118.3	107.7	148.0
182	161.7	80.6	88.2	83.4	81.7	75.6	123.1	119.7	108.9	149.7
184	163.4	81.5	89.2	84.4	82.7	76.6	124.3	121.0	110.1	151.4
186	165.2	82.5	90.2	85.3	83.7	77.6	125.8	122.4	111.3	153.0
188	167.0	83.4	91.2	86.3	84.6	78.5	127.2	123.7	112.5	154.7
190	168.8	84.3	92.2	87.2	85.6	79.5	128.5	125.1	113.8	156.4
192	170.5	85.3	93.2	88.2	86.6	80.5	129.9	126.4	115.0	158.0
194	172.3	86.2	94.2	89.2	87.6	81.4	131.3	127.8	116.2	159.7
196	174.1	87.1	95.2	90.1	88.5	82.4	132.7	129.2	117.4	161.4
198	175.9	88.1	96.2	91.1	89.5	83.4	134.1	130.5	118.6	163.0
200	177.7	89.0	97.2	92.0	90.5	84.4	135.4	131.9	119.8	164.7
202	179.4	89.9	98.3	93.0	91.4	85.3	136.8	133.2	121.0	166.4
204	181.2	90.9	99.3	94.0	92.4	86.3	138.2	134.6	122.3	168.0
206	183.0	91.8	100.3	94.9	93.4	87.3	139.6	135.9	123.5	169.7
208	184.8	92.8	101.3	95.9	94.4	88.3	141.0	137.3	124.7	171.4
210	186.5	93.7	102.3	96.9	95.4	89.2	142.3	138.6	126.0	173.0
212	188.3	94.6	103.3	97.8	96.3	90.2	143.7	140.0	127.2	174.7
214	190.1	95.6	104.3	98.8	97.3	91.2	145.1	141.4	128.4	176.4
216	191.9	96.5	105.3	99.8	98.3	92.2	146.5	142.7	129.6	178.0
218	193.6	97.5	106.4	100.8	99.3	93.2	147.9	144.1	130.9	179.7
220	195.4	98.4	107.4	101.7	100.3	94.2	149.3	145.4	132.1	181.4
222	197.2	99.4	108.4	102.7	101.2	95.1	150.7	146.8	133.3	183.0
224	199.0	100.3	109.4	103.7	102.2	96.1	152.0	148.1	134.5	184.7
226	200.7	101.3	110.4	104.6	103.2	97.1	153.4	149.5	135.8	186.4
228	202.5	102.2	111.4	105.6	104.2	98.1	154.8	150.8	137.0	188.0
230	204.3	103.2	112.5	106.6	105.2	99.1	156.2	152.2	138.2	189.7
232	206.1	104.1	113.5	107.6	106.2	100.1	157.6	153.6	139.4	191.3
234	207.9	105.1	114.5	108.6	107.2	101.1	159.0	154.9	140.7	193.0
236	209.6	106.0	115.5	109.5	108.2	102.1	160.3	156.3	141.9	194.7
238	211.4	107.0	116.5	110.5	109.2	103.1	161.7	157.6	143.2	196.3
240	213.2	108.0	117.6	111.5	110.1	104.0	163.1	159.0	144.4	198.0
242	215.0	108.9	118.6	112.5	111.1	105.0	164.5	160.3	145.6	199.7
244	216.7	109.9	119.6	113.5	112.1	106.0	165.9	161.7	146.9	201.3
246	218.5	110.8	120.6	114.5	113.1	107.0	167.3	163.1	148.1	203.0
248	220.3	111.8	121.7	115.4	114.1	108.0	168.7	164.4	149.3	204.7
250	222.1	112.8	122.7	116.4	115.1	109.0	170.1	165.8	150.6	206.3
252	223.8	113.7	123.7	117.4	116.1	110.0	171.5	167.2	151.8	208.0
254	225.6	114.7	124.7	118.4	117.1	111.0	172.8	168.5	153.1	209.7
256	227.4	115.7	125.8	119.4	118.1	112.0	174.2	169.9	154.3	211.3
258	229.2	116.6	126.8	120.4	119.1	113.0	175.6	171.3	155.5	213.0
260	231.0	117.6	127.8	121.4	120.1	114.0	177.0	172.6	156.8	214.7
262	232.7	118.6	128.9	122.4	121.1	115.0	178.4	174.0	158.0	216.3
264	234.5	119.5	129.9	123.4	122.1	116.0	179.8	175.3	159.3	218.0
266	236.3	120.5	130.9	124.4	123.1	117.0	181.2	176.7	160.5	219.7
268	238.1	121.5	131.9	125.4	124.1	118.0	182.6	178.1	161.8	221.3

Sugars from Cuprous Oxide and Copper (Munson and Walker-Hammond-Given)—*Continued*

Cu_2O	Cu	Dextrose	Levulose	Invert Sugar	Invert Sugar in Presence of Sucrose: 0.4 g. total sugar	Invert Sugar in Presence of Sucrose: 2 g. total sugar	Lactose $C_{12}H_{22}O_{11} \cdot H_2O$	Lactose in Presence of Sucrose: 1 : 4	Lactose in Presence of Sucrose: 1 : 12	Maltose $C_{12}H_{22}O_{11} \cdot H_2O$
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
270	239.8	122.5	133.0	126.4	125.1	119.0	184.0	179.4	163.0	223.0
272	241.6	123.4	134.0	127.4	126.2	120.0	185.3	180.8	164.3	224.6
274	243.4	124.4	135.0	128.4	127.2	121.1	186.7	182.2	165.5	226.3
276	245.2	125.4	136.1	129.4	128.2	122.1	188.1	183.5	166.8	228.0
278	246.9	126.4	137.1	130.4	129.2	123.1	189.5	184.9	168.0	229.6
280	248.7	127.3	138.2	131.4	130.2	124.1	190.9	186.3	169.3	231.3
282	250.5	128.3	139.2	132.4	131.2	125.1	192.3	187.6	170.5	233.0
284	252.3	129.3	140.2	133.4	132.2	126.1	193.7	189.0	171.8	234.6
286	254.0	130.3	141.3	134.4	133.2	127.1	195.1	190.4	173.0	236.3
288	255.8	131.3	142.3	135.4	134.3	128.1	196.5	191.7	174.3	238.0
290	257.6	132.3	143.4	136.4	135.3	129.2	197.8	193.1	175.5	239.6
292	259.4	133.2	144.4	137.4	136.3	130.2	199.2	194.4	176.8	241.3
294	261.2	134.2	145.4	138.4	137.3	131.2	200.6	195.8	178.1	242.9
296	262.9	135.2	146.5	139.4	138.3	132.2	202.0	197.2	179.3	244.6
298	264.7	136.2	147.5	140.5	139.4	133.2	203.4	198.6	180.6	246.3
300	266.5	137.2	148.6	141.5	140.4	134.2	204.8	199.9	181.8	247.9
302	268.3	138.2	149.6	142.5	141.4	135.3	206.2	201.3	183.1	249.6
304	270.0	139.2	150.6	143.5	142.4	136.3	207.6	202.7	184.4	251.3
306	271.8	140.2	151.7	144.5	143.4	137.3	209.0	204.0	185.6	252.9
308	273.6	141.2	152.8	145.5	144.5	138.3	210.4	205.4	186.9	254.6
310	275.4	142.2	153.8	146.6	145.5	139.4	211.8	206.8	188.1	256.3
312	277.1	143.2	154.9	147.6	146.5	140.4	213.2	208.1	189.4	257.9
314	278.9	144.2	155.9	148.6	147.6	141.4	214.6	209.5	190.7	259.6
316	280.7	145.2	157.0	149.6	148.6	142.4	216.0	210.9	191.9	261.2
318	282.5	146.2	158.0	150.7	149.6	143.5	217.3	212.2	193.2	262.9
320	284.2	147.2	159.1	151.7	150.7	144.5	218.7	213.6	194.4	264.6
322	286.0	148.2	160.1	152.7	151.7	145.5	220.1	215.5	195.7	266.2
324	287.8	149.2	161.2	153.7	152.7	146.6	221.5	216.4	197.0	267.9
326	289.6	150.2	162.2	154.8	153.8	147.6	222.9	217.7	198.2	269.6
328	291.4	151.2	163.3	155.8	154.8	148.6	224.3	219.1	199.5	271.2
330	293.1	152.2	164.3	156.8	155.8	149.7	225.7	220.5	200.8	272.9
332	294.9	153.2	165.4	157.9	156.9	150.7	227.1	221.8	202.0	274.6
334	296.7	154.2	166.4	158.9	157.9	151.7	228.5	223.2	203.3	276.2
336	298.5	155.2	167.5	159.9	159.0	152.8	229.9	224.6	204.6	277.9
338	300.2	156.3	168.6	161.0	160.0	153.8	231.3	226.0	205.9	279.5
340	302.0	157.3	169.6	162.0	161.0	154.8	232.7	227.4	207.1	281.2
342	303.8	158.3	170.7	163.1	162.1	155.9	234.1	228.7	208.4	282.9
344	305.6	159.3	171.7	164.1	163.1	156.9	235.5	230.1	209.7	284.5
346	307.3	160.3	172.8	165.1	164.2	158.0	236.9	231.5	211.0	286.2
348	309.1	161.4	173.9	166.2	165.2	159.0	238.3	232.9	212.2	287.9
350	310.9	162.4	174.9	167.2	166.3	160.1	239.7	234.3	213.5	289.5
352	312.7	163.4	176.0	168.3	167.3	161.1	241.1	235.6	214.8	291.2
354	314.4	164.4	177.1	169.3	168.4	162.2	242.5	237.0	216.1	292.8
356	316.2	165.4	178.1	170.4	169.4	163.2	243.9	238.4	217.3	294.5
358	318.0	166.5	179.2	171.4	170.5	164.3	245.3	239.8	218.6	296.2

Reducing Sugars from Cuprous Oxide and Copper (Munson and Walker-Hammond-Given)—*Continued*

Cu_2O	Cu	Dextrose	Levulose	Invert Sugar	Invert Sugar in Presence of Sucrose		Lactose $C_{12}H_{22}O_{11} \cdot H_2O$	Lactose in Presence of Sucrose		Maltose $C_{12}H_{22}O_{11} \cdot H_2O$
					0.4 g. total sugar	2 g. total sugar		1 : 4	1 : 12	
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
360	319.8	167.5	180.2	172.5	171.5	165.3	246.7	241.2	219.9	297.8
362	321.6	168.5	181.3	173.5	172.6	166.4	248.1	242.5	221.2	299.5
364	323.3	169.6	182.4	174.6	173.7	167.4	249.5	243.9	222.5	301.2
366	325.1	170.6	183.5	175.6	174.7	168.5	250.9	245.3	223.7	302.8
368	326.9	171.6	184.5	176.7	175.8	169.5	252.3	246.7	225.0	304.5
370	328.7	172.7	185.6	177.7	176.8	170.6	253.7	248.1	226.3	306.1
372	330.4	173.7	186.7	178.8	177.9	171.6	255.1	249.5	227.6	307.8
374	332.2	174.7	187.7	179.8	179.0	172.7	256.5	250.9	228.9	309.5
376	334.0	175.8	188.8	180.9	180.0	173.7	257.9	252.2	230.2	311.1
378	335.8	176.8	189.9	182.0	181.1	174.8	259.3	253.6	231.5	312.8
380	337.5	177.9	191.0	183.0	182.1	175.9	260.7	255.0	232.8	314.5
382	339.3	178.9	192.1	184.1	183.2	176.9	262.1	256.4	234.1	316.1
384	341.1	180.0	193.1	185.2	184.3	178.0	263.5	257.8	235.4	317.8
386	342.9	181.0	194.2	186.2	185.4	179.1	264.9	259.2	236.6	319.4
388	344.6	182.0	195.3	187.3	186.4	180.1	266.5	260.5	237.9	321.1
390	346.4	183.1	196.4	188.4	187.5	181.2	267.7	261.9	239.2	322.8
392	348.2	184.1	197.4	189.4	188.6	182.3	269.1	263.3	240.5	324.4
394	350.0	185.2	198.5	190.5	189.7	183.3	270.5	264.7	241.8	326.1
396	351.8	186.2	199.6	191.6	190.7	184.4	271.9	266.1	243.1	327.7
398	353.5	187.3	200.7	192.7	191.8	185.5	273.3	267.5	244.4	329.4
400	355.3	188.4	201.8	193.7	192.9	186.5	274.7	268.9	245.7	331.1
402	357.1	189.4	202.9	194.8	194.0	187.6	276.1	270.3	247.0	332.7
404	358.9	190.5	204.0	195.9	195.0	188.7	277.5	271.7	248.3	334.4
406	360.6	191.5	205.0	197.0	196.1	189.8	278.9	273.0	249.6	336.0
408	362.4	192.6	206.1	198.1	197.2	190.8	280.3	274.4	251.0	337.7
410	364.2	193.7	207.2	199.1	198.3	191.9	281.7	275.8	252.3	339.4
412	366.0	194.7	208.3	200.2	199.4	193.0	283.2	277.2	253.6	341.0
414	367.7	195.8	209.4	201.3	200.5	194.1	284.6	278.6	254.9	342.7
416	369.5	196.8	210.5	202.4	201.6	195.2	286.0	280.0	256.2	344.4
418	371.3	197.9	211.6	203.5	202.6	196.2	287.4	281.4	257.5	346.0
420	373.1	199.0	212.7	204.6	203.7	197.3	288.8	282.8	258.8	347.7
422	374.8	200.1	213.8	205.7	204.8	198.4	290.2	284.2	260.1	349.3
424	376.6	201.1	214.9	206.7	205.9	199.5	291.6	285.6	261.4	351.0
426	378.4	202.2	216.0	207.8	207.0	200.6	293.0	287.0	262.7	352.7
428	380.2	203.3	217.1	208.9	208.1	201.7	294.4	288.4	264.0	354.3
430	382.0	204.4	218.2	210.0	209.2	202.7	295.8	289.8	265.4	356.0
432	383.7	205.5	219.3	211.1	210.3	203.8	297.2	291.2	266.6	357.6
434	385.5	206.5	220.4	212.2	211.4	204.9	298.6	292.6	268.0	359.3
436	387.3	207.6	221.5	213.3	212.5	206.0	300.0	294.0	269.3	361.0
438	389.1	208.7	222.6	214.4	213.6	207.1	301.4	295.4	270.6	362.6
440	390.8	209.8	223.7	215.5	214.7	208.2	302.8	296.8	272.0	364.3
442	392.6	210.9	224.8	216.6	215.8	209.3	304.2	298.2	273.3	365.9
444	394.4	212.0	225.9	217.8	216.9	210.4	305.6	299.6	274.6	367.6
446	396.2	213.1	227.0	218.9	218.0	211.5	307.0	301.0	275.9	369.3
448	397.9	214.1	228.1	220.0	219.1	212.6	308.4	302.4	277.2	370.9

Reducing Sugars from Cuprous Oxide and Copper (Munson and Walker-Hammond-Given)—*Concluded*

Cu_2O	Cu	Dextrose	Levulose	Invert Sugar	Invert Sugar in Presence of Sucrose: 0.4 g. total sugar	Invert Sugar in Presence of Sucrose: 2 g. total sugar	Lactose $C_{12}H_{22}O_{11} \cdot H_2O$	Lactose in Presence of Sucrose: 1 : 4	Lactose in Presence of Sucrose: 1 : 12	Maltose $C_{12}H_{22}O_{11} \cdot H_2O$
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
450	399.7	215.2	229.2	221.1	220.2	213.7	309.9	303.8	278.6	372.6
452	401.5	216.3	230.4	222.2	221.4	214.8	311.3	305.2	279.9	374.2
454	403.3	217.4	231.5	223.3	222.5	215.9	312.7	306.6	281.2	375.9
456	405.1	218.5	232.6	224.4	223.6	217.0	314.1	308.0	282.5	377.6
458	406.8	219.6	233.7	225.5	224.7	218.1	315.5	309.4	283.9	379.2
460	408.6	220.7	234.9	226.7	225.8	219.2	316.9	310.8	285.2	380.9
462	410.4	221.8	236.0	227.8	226.9	220.3	318.3	312.2	286.5	382.5
464	412.2	222.9	237.2	228.9	228.1	221.4	319.7	313.6	287.8	384.2
466	413.9	224.0	238.3	230.0	229.2	222.5	321.1	315.0	289.2	385.9
468	415.7	225.1	239.5	231.2	230.3	223.7	322.5	316.4	290.5	387.5
470	417.5	226.2	240.6	232.3	231.4	224.8	323.9	317.7	291.8	389.2
472	419.3	227.4	241.8	233.4	232.5	225.9	325.3	319.1	293.2	390.8
474	421.0	228.3	242.9	234.5	233.7	227.0	326.8	320.5	294.5	392.5
476	422.8	229.6	244.1	235.7	234.8	228.1	328.2	321.9	295.8	394.2
478	424.6	230.7	245.3	236.8	235.9	229.2	329.6	323.3	297.1	395.8
480	426.4	231.8	246.6	237.9	237.1	230.3	331.0	324.7	298.5	397.5
482	428.1	232.9	247.9	239.1	238.2	231.5	332.4	326.1	299.8	399.1
484	429.9	234.1	249.1	240.2	239.3	232.6	333.8	327.5	301.1	400.8
486	431.7	235.2	250.6	241.4	240.5	233.7	335.2	328.9	302.5	402.4
488	433.5	236.3	252.1	242.5	241.6	234.8	336.6	330.3	303.8	404.1
490	435.3	237.4	253.9	243.6	242.7	236.0	338.0	331.7	305.1	405.8

Gravimetric Copper Reduction Methods. **Munson and Walker Unified Gravimetric Method.**[41] In this general or unified method the same analytical process serves for the determination of dextrose, invert sugar (alone and in the presence of sucrose), lactose, and maltose, the weights of each, corresponding to the weights of copper and cuprous oxide, appearing in the same table. The Fehling-Soxhlet reagent is employed, as in most copper reduction methods, the Allihn method being a notable exception.

Apparatus. *Gooch Crucibles,* provided with long-fiber asbestos treated as follows:

Digest with 1 + 3 hydrochloric acid for 2 or 3 days, pour off the acid, and wash with hot water. Next digest the asbestos in like manner with 25% sodium hydroxide solution, then treat for several hours with hot Fehling-Soxhlet solution, and finally, after washing free from alkali, boil with 1 + 3 nitric acid, and wash thoroughly with hot water. Reduce to a uniform disintegrated mass by cutting and scraping. Shake with water to a pulp when needed.

It is of the utmost importance that the asbestos be of such a quality as to retain the very fine cuprous oxide. Pack the crucible with a layer about 1 cm. thick, pressed down so as to form a compact mat. Wash thoroughly with water to remove fine particles, then with ethanol and ether. Dry 30 minutes in a boiling water oven, cool in a desiccator, and weigh.

The originators recommend dissolving the cuprous oxide, after the determination, with nitric acid, washing thoroughly, drying, and weighing as before for a new determination.

REAGENTS. *Fehling-Soxhlet Reagent.* Solutions 1 and 2, prepared as described above.

PROCESS. *Copper Reduction.* Mix in a 400 ml. beaker 15 ml. each of *Fehling-Soxhlet solutions 1 and 2,* then run in from a pipet 50 ml. of the sugar solution (or if a smaller volume is used, dilute the whole to 100 ml.) containing not more than 0.5% of reducing sugar. Heat to boiling over asbestos gauze so that boiling begins in exactly 4 minutes and continue the boiling exactly 2 minutes longer, keeping the beaker covered with a watch-glass.

Filtration. Without delay filter on the Gooch crucible, which in the meantime has been heated, cooled, and weighed. After pouring off the clear supernatant liquid onto the crucible, remove the clear filtrate so that if any cuprous oxide runs through later it will be evident. Wash with water at about 60°, then with 10 ml. of *ethanol,* and finally with 10 ml. of *ether.*

Weighing as Cuprous Oxide. Dry 30 minutes in a boiling water oven, cool in a desiccator, and weigh as cuprous oxide.

Weighing as Metallic Copper. If desired, the cuprous oxide may be reduced to metallic copper by heating cautiously in a current of *hydrogen,* passed through a Rose perforated unglazed cover, through a porcelain tube, cooling in the same gas.

Weighing as Cupric Oxide. Ignition in the open air to the black oxide (cupric oxide) may also be practiced, in which case multiply the weight of the precipitate by 0.79892 to obtain the metallic copper, since weights of cupric oxide are not given in the Munson and Walker table.

CALCULATION. See Munson and Walker-Hammond-Given table.

Browne and Zerban [42] give the complete Hammond table with columns headed glucose (dextrose), invert sugar, and invert sugar and sucrose (total sugar 0.3, 0.4, and 2.0 g.), as well as fructose (levulose). The values for concentrations less than 20 mg. of reducing sugar, being extrapolated, are only approximate.

Quisumbing and Thomas Unified Copper Reduction Gravimetric Method.[43] REAGENTS. *Cupric Sulfate Solution.* Make a hot saturated solution of $CuSO_4 \cdot 5H_2O$, washed free from surface impurities with water, and filter. Determine the copper (Cu) electrolytically and dilute so that 25 ml. will contain 525 mg. of metallic copper, equivalent to 41.2 g. of $CuSO_4 \cdot 5H_2O$ in 500 ml. of solution.

Alkaline Tartrate Solution. Dissolve NaOH, purified by ethanol, to saturation, siphon off the clear solution after several days, and determine its alkalinity by titration. Dissolve 173 g. of $KNaC_4H_4O_6 \cdot 4H_2O$ in a 500-ml. graduated flask, add NaOH solution equivalent to 65 g. of sodium hydroxide and dilute to 500 ml.

PROCESS. Pipet 25 ml. each of the *copper sulfate* and *alkaline tartrate solutions* into a 400-ml. beaker (9 cm. in diameter), add 50 ml. of the sugar solution, containing 50 to 150 mg. of dextrose, levulose, or invert sugar, or 100 to 300 mg. of lactose or maltose, cover with a watch-glass, and heat in a water bath at 80° exactly 30 minutes. Immediately filter on a Gooch crucible, wash the precipitate with water, and weigh the copper as the metal or suboxide.

CALCULATION. See table below.

Other Unified Gravimetric Methods. As early as 1896 Kjeldahl [44] in Denmark and Defren and O'Sullivan [45] in the United States described unified methods, each with calculation tables. Kjeldahl in his table gives weights of dextrose, levulose, invert sugar, galactose, lactose, and maltose equivalent to copper and cupric oxide; Defren and O'Sullivan limited the sugars to dextrose, maltose, and lactose and weighed only as cupric oxide. Woy [46] modified the Kjeldahl method and

Reducing Sugars from Cuprous Oxide and Copper (Quisumbing and Thomas)

Cu_2O	Cu	Dextrose	Levulose	Invert Sugar	Lactose		Maltose	
					$C_{12}H_{22}O_{11}$	$C_{12}H_{22}O_{11} \cdot H_2O$	$C_{12}H_{22}O_{11}$	$C_{12}H_{22}O_{11} \cdot H_2O$
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
11.1	10	4.8	5.3	5.0	7.7	8.1	9.4	9.9
22.5	20	9.5	10.5	10.1	15.5	16.3	18.8	19.8
33.8	30	14.3	15.8	15.2	23.2	24.4	28.2	29.7
45.0	40	19.1	21.2	20.3	30.9	32.5	37.6	39.6
56.3	50	24.0	26.5	25.4	38.7	40.7	47.0	49.5
67.6	60	28.9	31.9	30.6	46.4	48.8	56.4	59.4
78.8	70	33.7	37.2	35.7	54.0	56.9	65.8	69.3
90.1	80	38.7	42.6	40.9	61.7	65.0	75.2	79.2
101.3	90	43.6	48.0	46.1	69.5	73.2	84.6	89.1
112.6	100	48.6	53.4	51.3	77.2	81.3	94.0	99.0
123.8	110	53.5	58.8	56.5	85.0	89.5	103.4	108.9
135.1	120	58.5	64.3	61.8	92.7	97.6	112.8	118.8
146.4	130	63.6	70.7	67.0	100.4	105.7	122.2	128.7
157.6	140	68.6	75.2	72.3	108.2	113.9	131.6	138.6
168.9	150	73.7	80.7	77.6	116.0	122.0	141.0	148.5
180.1	160	78.8	86.2	82.9	123.7	130.1	150.4	158.4
191.4	170	83.9	91.7	88.3	131.4	138.3	159.8	168.3
202.6	180	89.1	97.2	93.7	139.1	146.4	169.2	178.2
213.9	190	94.2	102.8	99.1	146.9	154.6	178.8	188.1
225.2	200	99.4	108.4	104.4	154.6	162.7	188.2	198.0
236.4	210	104.6	114.0	109.8	162.3	170.9	197.6	207.9
247.7	220	109.9	119.6	115.2	170.0	179.0	207.0	217.8
258.9	230	115.1	125.2	120.6	177.8	187.2	216.4	227.7
270.2	240	120.4	130.8	126.1	185.5	195.3	225.8	237.6
281.5	250	125.7	136.4	131.6	193.2	203.4	235.2	247.5
292.7	260	131.0	142.1	137.1	201.0	211.6	244.6	257.4
304.0	270	136.4	147.8	142.6	208.8	219.8	254.0	267.3
315.2	280	141.7	153.5	148.2	216.5	227.9	263.4	277.2
326.5	290	147.1	159.2	153.7	224.2	236.0	272.8	287.1
337.8	300	152.6	165.0	159.3	232.0	244.2	282.2	297.0
349.0	310	158.0	170.7	164.9	239.7	252.3	291.6	306.9
360.3	320	163.5	176.5	170.5	247.5	260.5	301.0	316.8
371.5	330	168.9	182.3	176.1	255.3	268.7	310.4	326.7
382.8	340	174.5	188.1	181.8	263.0	276.8	319.8	336.6
394.0	350	180.0	193.9	187.4	270.7	285.0	329.2	346.5
405.3	360	185.5	199.7	193.1	278.4	293.1	338.6	356.4
416.6	370	191.1	205.5	198.8	286.2	301.3	348.0	366.3
427.8	380	196.7	211.4	204.5	293.9	309.4	357.4	376.2
439.1	390	202.3	217.3	210.2	301.6	317.5	366.8	386.1
450.3	400	208.0	223.2	216.0	309.4	325.7	376.2	396.0
461.6	410	213.7	229.1	221.8	317.1	333.8	385.6	405.9
472.9	420	219.4	235.0	227.6	324.9	342.0	395.0	415.8
484.1	430	225.1	240.9	233.4	332.6	350.1	404.4	425.7
495.4	440	230.8	246.9	239.2	340.4	358.3	413.8	435.6
506.6	450	236.6	252.9	245.0	348.1	366.4	423.2	445.5
517.9	460	242.4	258.9	250.9	355.9	374.6	432.6	455.4
529.1	470	248.1	264.9	256.8	363.6	382.7	442.0	465.3
540.4	480	250.8	270.9	262.7	371.3	390.9	451.4	475.2

Jessen-Hansen[47] further modified it so as to include in a special table weights of invert sugar in solution alone and with 0.15, 2.0, and 10.0 g. of sucrose.

All the above authors carried out the reduction in a boiling water bath which was believed to furnish a more uniform heating than over a flame. Conversion into the black oxide (cupric oxide) has the advantage of burning any organic impurity present in the cuprous oxide. Complete descriptions of the Kjeldahl method and its modifications, together with tables, are given by Browne and Zerban.[48] The Defren and O'Sullivan method and tables are given by Leach and Winton.[49]

Zerban and Sattler Unified Calculation Method.[50] Extending the methods of Zerban[51] and of Zerban and Wiley[52] for dextrose and levulose in raw cane sugar, and of Erb and Zerban[53] for the two sugars in cane molasses, Zerban and Sattler (New York Sugar Trade Laboratory) elaborated the details so as to include four sugars.

Total reducing sugars are determined by Fehling solution, the monosaccharides by Barfoed's reagent as modified by Steinhoff,[54] levulose by the Jackson and Mathews Modification (which see) of the Nijns Method, and lactose by oxidation to mucic acid, or preferably by copper reduction after fermenting the other sugars by a modification of the Hoffman, Schweitzer, and Dalby Method.[55]

The results of the analyses are calculated by a series of approximations until finally two successive calculations give practically identical results.

Zerban and Sattler in their paper give the four formulas, the calculation tables, and the somewhat complicated procedure for obtaining the necessary correction.

***Ferricyanide Volumetric Methods.* Gentele Method.** The determination of sugars by the reaction involving the reduction of potassium ferricyanide to ferrocyanide in alkaline solution was first practiced by Gentele.[56] In the original method, the solution (40 ml.), containing 1 g. of refined or crude sugar or sirup, is warmed to 70° and titrated directly with *standard alkaline ferricyanide solution* (10.980 g. of potassium ferricyanide and 5.5 g. of potassium hydroxide in 1 liter) until the yellow color persists after stirring 5 seconds. It is stated that oxalic and tartaric acids interfere, but citric, succinic, and acetic acids do not.

I. The Hagedorn and Jensen Modification[57] has been much used in blood analysis.

II. Whitmoyer Modification for Reducing Sugar, Dextrose, or Levulose.[58] Advantage was taken by Whitmoyer of the discovery of Furman and Evans[59] that the ferrocyanide formed in the reaction with the sugar may be quantitatively oxidized by standard ceric sulfate solution. A sharp end-point is obtained by the use of alphazurine G as an inside indicator.

Reagents. *Potassium Ferricyanide Solution,* 0.8%. Dissolve 8 g. of $K_3Fe(CN)_6$ in water and dilute to 1 liter at 20°. If stored in a Pyrex bottle in a dark place, the solution keeps at least 6 weeks.

Standard Ceric Sulfate Solution, 0.01 *M*. The salt, $Ce(SO_4)_2 \cdot 4H_2O$, is obtainable from supply houses or may be made in the laboratory from cerous oxalate, $Ce_2(C_2O_4)_3 \cdot 9H_2O$, or ceric oxide, CeO_2, as described by Willard and Young.[60] Prepare a stock solution of 0.08122 *M* ceric sulfate and dilute 750 ml. of the solution and 300 ml. of H_2SO_4 to 6 liters. Standardize against 1.5 mg. of U. S. Bureau of Standards dextrose dissolved in 100 ml. of water, following the method as described below or against 15 ml. of a solution of 0.36 g. $K_4Fe(CN)_6 \cdot 3H_2O$ in 250 ml. of water, after dilution to 95 ml. and acidifying with 5.7 ml. of 4.3 *M* H_2SO_4, using as indicator 5 drops of 0.4% alphazurine G solution.

Alphazurine G Solution, 0.4%. The dye furnished by the National Aniline Co. is suitable.

Sodium Carbonate Solution, 14%. Dis-

solve 140 g. of $Na_2CO_3 \cdot H_2O$ in water and make up to 1 liter.

PROCESS. The modification here described is for solutions containing 0.5 to 2.0 mg. of dextrose, levulose, or invert sugar per 100 ml. A micro method for solutions containing 0.01 to 0.1 mg. per kilo is described in Whitmoyer's paper.

Ferricyanide Reduction. Place 5 ml. of *14% sodium carbonate solution* and 15 ml. of water in a 125-ml. flat-bottom Pyrex flask, mix, and add 5 ml. of *0.8% potassium ferricyanide solution.* Allow to stand at 25° for 4.5 minutes, then add from a pipet, 1.5 minutes before placing in the water bath, 100 ml. of the sugar solution. Insert a small rod in the mixture, stir, and cover the flask with a stemless funnel. Place the mixture in a water bath kept at 80° and after allowing to stand 10 seconds stir for 1 minute. After 30 minutes cool by immersing the flask in water at 25° (allowing 30 seconds for the change) and after 5 seconds stir for 1 minute and keep 4.5 additional minutes in the bath.

Ceric Sulfate Titration. Pour the cooled mixture into a 400-ml. beaker and rinse the flask three times with 5-ml. portions of water. Acidify with 5.7 ml. of *4.3 M sulfuric acid,* then titrate with *0.01 M ceric sulfate solution,* using a Folin micro buret to which is attached a jet-tube that permits the delivery of drops as small as 0.015 ml. Add the drops in rapid succession, with constant shaking, up to about 0.1 ml. of the end-point, then add 5 drops of *0.4% alphazurine G solution.* Complete the titration, adding the *ceric sulfate solution* in fractions of a drop with shaking until the color changes from yellow-green to brown.

CALCULATION. Between the limits of 0.5 and 2.0 mg., Whitmoyer found that each 0.2 mg. of invert sugar treated by the above method was equivalent to 0.545 ml. of 0.01 *M* ceric sulfate solution. Consequently, for use in the calculation 1 ml. of 0.01 *M* ceric sulfate solution is equivalent to 0.367 mg. of invert sugar. The results within the same limits and the same method were practically the same for levulose and dextrose separately treated as for invert sugar; hence the factor 0.367 applies to all three sugars, but obviously the procedure does not serve for the determination of dextrose, levulose, or invert sugar in the presence of one or both of the other two.

The presence of 18 mg. of sucrose in the solution caused an increase of only about 0.02 ml. of 0.01 *M* ceric sulfate solution, regardless of the concentration of dextrose, levulose, or invert sugar between the limits of 0.5 and 2.0 mg. For many practical purposes this increase due to sucrose is negligible.

III. Hassid Modification for Reducing Sugar.[61] This rapid modification was devised at the University of California.

REAGENTS. *Ferricyanide Solution,* alkaline. Dissolve 8.25 g. of $K_3Fe(CN)_6$ and 10.6 g. of sodium carbonate in cold water and make up to 1 liter. Store in a dark bottle in a cool place.

Setopaline C Indicator. Dissolve 0.1 g. in 100 ml. of water.

Ceric Sulfate Solution, 0.01 *N.* Prepare an approximately 0.25 *N* stock solution by dissolving 75.5 g. of $Ce(SO_4)_2 \cdot 4H_2O$ (55% pure) in a mixture of 300 ml. of water and 30 ml. of H_2SO_4 with the aid of heat, filter, cool, and make up to 500 ml. Standardize by titrating 15 ml., diluted with 50 ml. of water and 3 ml. of 1 + 1 H_2SO_4, with 0.1 *N* Mohr salt solution (39.214 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ dissolved in a mixture of 300 ml. of water and 25 ml. of 1 + 1 H_2SO_4 and diluted to 1 liter) until nearly colorless, then add 8 drops of Setopaline C indicator and continue the titration to a sharp change from golden brown to light yellow.

For use, dilute 20 ml. of the stock solution and 50 ml. of 1 + 1 H_2SO_4 to 500 ml. Standardize against 5-ml. aliquots of a 0.05 to 0.07% solution of pure dextrose (prepared from a stock 1% solution in 0.25% benzoic

acid), and adjust to exactly 0.01 *N;* 1 mg. of dextrose = 3 ml. of 0.01 *N* $Ce(SO_4)_2 \cdot 4H_2O$.

PROCESS. *Extraction and Clarification.* Extract 1 to 2 g. of the dry material for 6 hours with 125 ml. of *80% ethanol* in a continuous extractor. Evaporate the extract on a steam bath to 10 ml., cool to room temperature, and clear with 5 ml. of *saturated lead acetate solution.* Delead with 10 ml. of *saturated disodium phosphate solution* and shake with 0.3 g. of *charcoal* (carboraffin) at intervals for 30 minutes. Filter through a Büchner funnel prepared with a thin layer of powdered *talc* over filter paper, rinse the flask with cold water, and finally wash the precipitate with warm water. Make up to 100 or 200 ml.

Ferricyanide Oxidation. Mix 5 ml. of the clarified solution, containing not more than 3.5 mg. of reducing sugar, with 5 ml. of *alkaline potassium ferricyanide solution* in a Pyrex test tube (145 x 28 mm.), then heat the tube in a boiling water bath for exactly 15 minutes.

Ceric Sulfate Titration. Cool under the tap to room temperature, add 5 ml. of *5 N sulfuric acid,* and shake. Add 7 to 10 drops of *Setopaline C indicator* and titrate with *0.01 N ceric sulfate* delivered from a 10-ml. buret until a golden brown color appears.

IV. Englis and Becker Modification for Levulose. The selective action of levulose in the presence of dextrose with potassium ferricyanide has been studied by Englis and Becker [62] and Becker and Englis [63] have adjusted the reagents and process for the determination of levulose up to 90 mg.

REAGENTS. *Alkaline Ferricyanide Reagent.* Dissolve 50 g. of $K_3Fe(CN)_6$, 225 g. of $Na_2HPO_4 \cdot 12H_2O$, and 150 g. of Na_2CO_3 in water and make up to 1 liter. If stored in a bottle painted black or if kept in a dark room, the solution, after aging 6 to 8 days, will keep at least a month.

PROCESS. Mix 10 ml. of the sugar solution, containing not more than 90 mg. of levulose or its equivalent in a levulose-dextrose mixture, and 25 ml. of the *alkaline ferricyanide reagent.* Heat at 50° in a water bath for 60 minutes, then cool by immersing the flask in cold water and carefully acidify with 60 ml. of *3 N sulfuric acid,* add 6 to 8 drops of *0.005 M sodium diphenylamine sulfonate solution,* and titrate with *standard 0.10 to 0.15 N ceric sulfate solution.*

CALCULATION. Compare with a curve plotted from the results on known quantities of pure levulose as found by Becker and Englis as follows:

$Ce(SO_4)_2 \approx K_3Fe(CN)_6$ Reduced	Levulose	$Ce(SO_4)_2 \approx K_3Fe(CN)_6$ Reduced	Levulose
milliequiv.	mg.	milliequiv.	mg.
0.226	4.9	2.089	49.3
0.450	9.9	2.460	59.2
0.874	19.7	2.813	69.0
1.296	29.6	3.152	78.9
1.700	39.4	3.477	88.7

The milligrams of apparent levulose, thus obtained, subtracted from the milligrams of total reducing sugars, previously determined by a standard method, gives the milligrams of apparent dextrose. Divide the latter by the proper factor (see below) and subtract this correction from the milligrams of apparent levulose to obtain a new approximation. Subtract the new approximation for levulose from the milligrams of total reducing sugars and divide the difference (dextrose), as before, by the factor to obtain a second correction for levulose. Repeat the calculations until successive values remain constant, two or at least three approximations being sufficient.

In choosing the proper factor, subtract one-fifteenth of the milligrams of apparent dextrose from the milligrams of apparent levulose to obtain an estimation of the actual

levulose, provided the latter is close to 40 mg. or there is a large amount of dextrose.

V. Strepkov Iodometric Modification for Levulose.[64] The procedure for levulose and dextrose (below) was developed at the University of Samarkand, U.S.S.R.

REAGENTS. *Ferricyanide Reagent*, 0.005 *N*. Dissolve 1.65 g. of $K_3Fe(CN)_6$ and 80 g. of $Na_2HPO_4 \cdot 12H_2O$ in water and make up to 1 liter.

Starch Solution, 1% saturated with NaCl.

PROCESS. *Ferricyanide Oxidation*. With a micro pipet measure 1 ml. of the sugar solution, containing not over 0.5 mg. of dextrose, into a test tube, add 2 ml. of the *ferricyanide reagent*, close the tube, and keep for 2.5 hours at 60° in a water bath heated from 61 to 62°.

Iodine Titration. Cool the solution, acidify with *10% acetic acid*, then add from a micro buret 2 ml. of *standard 0.005 N iodine solution* and titrate back with *standard 0.005 N thiosulfate solution*, using *starch solution* as indicator.

LEVULOSE FROM SODIUM THIOSULFATE (STREPKOV)

0.005 *N* $Na_2S_2O_3$	0.01 ml. 0.005 *N* $Na_2S_2O_3$				
	0	2	4	6	8
ml.	mg. levulose				
0.30	0.02	0.04	0.07	0.09	0.11
0.40	0.13	0.16	0.18	0.20	0.22
0.50	0.24	0.27	0.29	0.31	0.33
0.60	0.36	0.38	0.40	0.42	0.44
0.70	0.47	0.49	0.51	0.53	0.56
0.80	0.58	0.60	0.62	0.64	0.67
0.90	0.69	0.71	0.73	0.76	0.78
1.00	0.80	0.82	0.84	0.87	0.89
1.10	0.91	0.93	0.96	0.98	1.00
1.20	1.02	1.04	1.07	1.09	1.11
1.30	1.13	1.16	1.18	1.20	1.22
1.40	1.24	1.27	1.29	1.31	1.33
1.50	1.36	1.38	1.40	1.42	1.44
1.60	1.47	1.49	....	....	....

Blank. Make a blank determination using 1 ml. of water and the same amount of reagents as in the actual analysis.

CALCULATION. Obtain the milligrams of levulose (L) by the following formula:

$$L = \frac{T - 0.28}{0.9}$$

in which T is the milliliters of thiosulfate used in the blank determination less that used in the actual analysis.

The calculation (other than the subtraction) is obviated by consulting the table in the preceding column.

VI. Strepkov Dichromate Modification for Dextrose.[65] REAGENTS. *Ferricyanide Reagent*, 0.02 *N*. Dissolve 6.6 g. of potassium ferricyanide and 40 g. of anhydrous sodium carbonate in water and make up to 1 liter.

Diphenylamine Indicator. Dilute to 1 liter with 5% H_2SO_4 15 ml. of a 0.2% solution of diphenylamine in H_2SO_4.

Standard Potassium Dichromate Solution. Dissolve 1.5925 g. of K_2CrO_7 in water and dilute to 1 liter.

PROCESS. Mix in a 250-ml. Erlenmeyer flask 20 ml. of the *ferricyanide reagent* with 10 ml. of the sugar solution containing not over 13.5 mg. of dextrose and heat on a boiling water bath for 15 minutes. Cool, add quickly 15 ml. of *diphenylamine indicator*, mix well, and titrate with *standard potassium dichromate solution*.

Conduct in like manner a blank determination, using 10 ml. of water, and introduce the correction.

CALCULATION. Use: 1 ml. of the standard K_2CrO_7 = 1 mg. of dextrose.

LEVULOSE AND DEXTROSE

Nyns Selective Copper Carbonate Volumetric Method.[66] In the original method and the form tentatively adopted by the

A.O.A.C., *standard permanganate* is used for the titration.

I. Jackson and Mathews Modification.[67] Standard 0.1573 *N* potassium dichromate solution is used for the titration.

Reagents. *Ost Solution.* Dissolve 250 g. of anhydrous K_2CO_3 in 700 ml. of hot water, add 100 g. of pulverized $KHCO_3$, and agitate until the solution is complete. Cool, add with vigorous agitation a solution of 25.3 g. of $CuSO_4 \cdot 5H_2O$ in 100 to 150 ml. of water, make up to 1 liter, and filter.

Standard Ferrous Ammonium Sulfate Solution, 0.1573 *N.* Dissolve 61.8 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in water, add 5 ml. of H_2SO_4, and make up to 1 liter. As there is a loss of about 0.3% of reducing power each day, titration is recommended at the beginning and end of each series of analyses.

Process. *Copper Reduction.* Place 50 ml. of *Ost solution* in a 150-ml. Erlenmeyer flask and add from a pipet a volume of the solution of the sample containing not more than 92 mg. of levulose or its equivalent of a levulose-dextrose mixture on the basis of dextrose having about one-twelfth the reducing power of levulose. Dilute with water to 70 ml. and digest for exactly 75 minutes, with rotary agitation every 10 to 15 minutes, in a water bath heated to exactly 55°.

At the expiration of the prescribed time, collect the precipitated cuprous oxide in a closely packed Gooch crucible, without attempting to remove completely the precipitate that adheres to the sides of the flask, and wash flask and precipitate thoroughly.

Dichromate Oxidation. Transfer the crucible together with the cuprous oxide and the asbestos mat to a 400-ml. beaker, add 5 to 10 ml. of water, and disintegrate the mat with a glass rod. Add from a buret *standard 0.1573 N potassium dichromate solution* sufficient to supply 3 to 4 ml. in excess of that required for the oxidation, using the first portion of about 1 ml. to dissolve the cuprous oxide adhering to the flask. Run into the Erlenmeyer flask 50 ml. of *1 + 1 hydrochloric acid* in such a manner as to bring it in contact with the adhering cuprous oxide and pour the acid slowly with constant stirring into the breaker. Continue the stirring until no more cuprous oxide can be seen from below through the bottom of the beaker. Rinse the Erlenmeyer flask with water into the beaker, add the crucible to dissolve any adhering cuprous oxide, remove the crucible with a rod, and dilute to 250 ml.

Ferrous Sulfate Titration. Titrate electrically the excess of dichromate with *standard ferrous sulfate solution.*

Alternate Gravimetric Procedure. If care is taken in the removal from the precipitation flask to the Gooch crucible, the cuprous oxide may be dried and weighed as such or, if impure, as cupric oxide after oxidation by ignition, or as metallic copper after reduction by ignition in a current of *hydrogen.*

Calculation. From the results on reducing sugars and the above procedure, calculate the levulose and dextrose by successive approximations as follows.

Obtain the content of apparent levulose in the Levulose from Copper Table herewith and that of the apparent dextrose by subtracting the content of apparent levulose from that of reducing sugars determined by the Lane and Eynon method. Divide the difference by 12.4 and subtract the quotient from the apparent levulose content, thus obtaining a new approximation for levulose and, by subtracting this from the reducing sugars, a new approximation for dextrose.

Again divide by 12.4 and proceed as before, repeating until the difference in two successive approximations is negligible.

This tedious calculation may be avoided by using the table headed Ratio of Levulose to Total Reducing Sugar herewith in which *T* is the buret reading by the Lane and Eynon titration and *l* is the apparent milligrams of levulose from the Levulose from Copper table.

Levulose from Copper (Jackson and Mathews)

Cu	Levulose	Cu	Levulose	Cu	Levulose	Cu	Levulose	Cu	Levulose	Cu	Levulose	Cu	Levulose	Cu	Levulose
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	0.6	40	13.9	79	25.1	118	36.0	157	46.6	196	56.8	235	67.9	274	80.4
2	1.1	41	14.2	80	25.4	119	36.2	158	46.9	197	57.1	236	68.2	275	80.7
3	1.6	42	14.5	81	25.7	120	36.5	159	47.1	198	57.3	237	68.5	276	81.0
4	2.1	43	14.8	82	25.9	121	36.8	160	47.4	199	57.6	238	68.8	277	81.4
5	2.5	44	15.1	83	26.2	122	37.1	161	47.7	200	57.9	239	69.1	278	81.7
6	2.9	45	15.4	84	26.5	123	37.3	162	47.9	201	58.1	240	69.4	279	82.0
7	3.3	46	15.7	85	26.8	124	37.6	163	48.2	202	58.4	241	69.7	280	82.4
8	3.7	47	16.0	86	27.0	125	37.9	164	48.4	203	58.7	242	70.0	281	82.7
9	4.1	48	16.3	87	27.3	126	38.2	165	48.7	204	58.9	243	70.3	282	83.1
10	4.5	49	16.6	88	27.6	127	38.5	166	49.0	205	59.2	244	70.7	283	83.4
11	4.8	50	16.8	89	27.9	128	38.7	167	49.2	206	59.4	245	71.0	284	83.8
12	5.1	51	17.1	90	28.1	129	39.0	168	49.5	207	59.7	246	71.3	285	84.1
13	5.5	52	17.4	91	28.4	130	39.3	169	49.7	208	60.0	247	71.6	286	84.4
14	5.9	53	17.7	92	28.7	131	39.6	170	50.0	209	60.3	248	71.9	287	84.8
15	6.2	54	18.0	93	29.0	132	39.9	171	50.2	210	60.6	249	72.2	288	85.1
16	6.5	55	18.3	94	29.2	133	40.1	172	50.5	211	60.9	250	72.5	289	85.5
17	6.9	56	18.6	95	29.5	134	40.4	173	50.8	212	61.1	251	72.8	290	85.9
18	7.2	57	18.9	96	29.8	135	40.7	174	51.0	213	61.4	252	73.1	291	86.2
19	7.6	58	19.1	97	30.1	136	40.9	175	51.3	214	61.7	253	73.5	292	86.6
20	7.9	59	19.4	98	30.4	137	41.2	176	51.5	215	62.0	254	73.8	293	86.9
21	8.2	60	19.7	99	30.7	138	41.5	177	51.8	216	62.3	255	74.1	294	87.3
22	8.5	61	20.0	100	30.9	139	41.7	178	52.1	217	62.6	256	74.4	295	87.6
23	8.9	62	20.3	101	31.2	140	42.0	179	52.3	218	62.9	257	74.7	296	88.0
24	9.2	63	20.6	102	31.5	141	42.3	180	52.6	219	63.2	258	75.1	297	88.4
25	9.5	64	20.9	103	31.8	142	42.6	181	52.8	220	63.4	259	75.4	298	88.7
26	9.8	65	21.2	104	32.1	143	42.8	182	53.1	221	63.7	260	75.7	299	89.1
27	10.1	66	21.4	105	32.3	144	43.1	183	53.4	222	64.0	261	76.0	300	89.5
28	10.4	67	21.7	106	32.6	145	43.4	184	53.6	223	64.3	262	76.4	301	89.8
29	10.7	68	22.0	107	32.9	146	43.7	185	53.9	224	64.6	263	76.7	302	90.2
30	11.0	69	22.2	108	33.2	147	43.9	186	54.2	225	64.9	264	77.0	303	90.5
31	11.3	70	22.5	109	33.5	148	44.2	187	54.4	226	65.2	265	77.4	304	90.9
32	11.6	71	22.8	110	33.7	149	44.5	188	54.7	227	65.5	266	77.7	305	91.3
33	11.9	72	23.1	111	34.0	150	44.7	189	54.9	228	65.8	267	78.1	306	91.7
34	12.2	73	23.4	112	34.3	151	45.0	190	55.2	229	66.1	268	78.4	307	92.0
35	12.5	74	23.7	113	34.6	152	45.3	191	55.5	230	66.4	269	78.7	308	92.4
36	12.8	75	24.0	114	34.8	153	45.6	192	55.7	231	66.7	270	79.0	309	92.8
37	13.1	76	24.2	115	35.1	154	45.8	193	56.0	232	67.0	271	79.4	310	93.2
38	13.4	77	24.5	116	35.4	155	46.1	194	56.3	233	67.3	272	79.7	311	93.5
39	13.7	78	24.8	117	35.7	156	46.4	195	56.5	234	67.6	273	80.0	312	93.9

RATIO OF LEVULOSE * TO TOTAL REDUCING SUGAR † (JACKSON AND MATHEWS)

$\frac{T \times l}{100}$	$T = 15$	$T = 25$	$T = 35$	$T = 45$	$\frac{T \times l}{100}$	$T = 15$	$T = 25$	$T = 35$	$T = 45$	$\frac{T \times l}{100}$	$T = 15$	$T = 25$	$T = 35$	$T = 45$
11	1.2	1.2	1.1	1.1	51	36.5	36.3	36.1	35.9	91	70.2	70.0	69.7	69.4
12	2.1	2.1	2.0	2.0	52	37.3	37.2	36.9	36.7	92	71.0	70.8	70.6	70.3
13	3.0	3.0	2.9	2.9	53	38.2	38.0	37.8	37.6	93	71.9	71.7	71.4	71.1
14	3.9	3.8	3.8	3.7	54	39.1	38.9	38.6	38.4	94	72.7	72.5	72.3	72.0
15	4.8	4.7	4.7	4.6	55	39.9	39.7	39.5	39.3	95	73.5	73.3	73.1	72.8
16	5.7	5.6	5.6	5.5	56	40.8	40.6	40.3	40.1	96	74.3	74.2	73.9	73.6
17	6.5	6.5	6.4	6.4	57	41.7	41.5	41.2	40.9	97	75.2	75.0	74.7	74.4
18	7.4	7.4	7.3	7.3	58	42.5	42.3	42.1	41.8	98	76.0	75.8	75.6	75.3
19	8.3	8.2	8.2	8.1	59	43.4	43.2	42.9	42.6	99	76.8	76.6	76.4	76.1
20	9.2	9.1	9.1	9.0	60	44.2	44.0	43.8	43.5	100	77.6	77.4	77.2	76.9
21	10.1	10.0	10.0	9.9	61	45.0	44.8	44.6	44.3	101	78.5	78.2	78.0	77.7
22	11.0	10.9	10.9	10.8	62	45.9	45.7	45.5	45.2	102	79.3	79.0	78.8	78.5
23	11.9	11.8	11.8	11.7	63	46.7	46.5	46.3	46.0	103	80.1	79.9	79.6	79.3
24	12.8	12.7	12.6	12.5	64	47.6	47.4	47.2	46.9	104	81.0	80.7	80.4	80.1
25	13.7	13.6	13.5	13.4	65	48.4	48.2	48.0	47.7	105	81.8	81.5	81.2	80.9
26	14.5	14.4	14.3	14.2	66	49.3	49.0	48.8	48.5	106	82.6	82.3	82.0	81.7
27	15.4	15.3	15.2	15.1	67	50.1	49.9	49.7	49.4	107	83.5	83.2	82.8	82.5
28	16.3	16.2	16.1	16.0	68	51.0	50.8	50.6	50.2	108	84.3	84.0	83.6	83.3
29	17.2	17.1	17.0	16.9	69	51.8	51.6	51.4	51.1	109	85.1	84.8	84.4	84.1
30	18.1	18.0	17.9	17.8	70	52.7	52.5	52.2	51.9	110	85.9	85.6	85.2	84.9
31	19.0	18.9	18.7	18.6	71	53.5	53.3	53.1	52.8	111	86.7	86.4	86.0	85.7
32	19.9	19.8	19.6	19.5	72	54.3	54.1	53.9	53.6	112	87.5	87.2	86.8	86.5
33	20.8	20.6	20.5	20.4	73	55.2	55.0	54.7	54.4	113	88.3	88.0	87.6	87.3
34	21.6	21.5	21.4	21.3	74	56.0	55.8	55.6	55.3	114	89.1	88.8	88.4	88.1
35	22.5	22.4	22.3	22.2	75	56.8	56.6	56.4	56.1	115	90.0	89.6	89.3	88.9
36	23.4	23.3	23.2	23.1	76	57.7	57.5	57.2	56.9	116	90.8	90.4	90.1	89.7
37	24.3	24.2	24.0	23.9	77	58.5	58.3	58.1	57.8	117	91.6	91.3	91.0	90.6
38	25.2	25.1	24.9	24.8	78	59.4	59.2	58.9	58.6	118	92.4	92.1	92.8	91.4
39	26.1	26.0	25.8	25.7	79	60.2	60.0	59.8	59.5	119	93.2	92.9	92.6	92.2
40	26.9	26.8	26.6	26.5	80	61.1	60.9	60.6	60.3	120	94.0	93.7	93.4	93.0
41	27.8	27.7	27.5	27.4	81	61.9	61.7	61.4	61.1	121	94.8	94.5	94.2	93.8
42	28.7	28.6	28.4	28.3	82	62.8	62.5	62.3	62.0	122	95.6	95.3	95.0	94.6
43	29.5	29.4	29.2	29.1	83	63.6	63.3	63.1	62.8	123	96.4	96.1	95.8	95.4
44	30.4	30.3	30.1	30.0	84	64.4	64.2	63.9	63.6	124	97.3	97.0	96.6	96.2
45	31.3	31.2	31.0	30.8	85	65.2	65.0	64.8	64.5	125	98.1	97.8	97.4	97.0
46	32.2	32.0	31.9	31.7	86	66.0	65.8	65.6	65.3	126	98.9	98.6	98.2	97.8
47	33.0	32.9	32.7	32.5	87	66.9	66.7	66.4	66.1	127	99.7	99.4	99.0	98.6
48	33.9	33.8	33.6	33.4	88	67.7	67.5	67.2	66.9					
49	34.8	34.6	34.4	34.2	89	68.5	68.3	68.1	67.8					
50	35.6	35.4	35.2	35.0	90	69.3	69.1	68.9	68.6					

* l = apparent levulose.
† T = total reducing sugars by Lane and Eynon.

II. Strepkov Iodometric Modification.[68] The reaction with the reduced copper, on which Strepkov (Samarkand, U.S.S.R.) depends, is as follows:

$$2Cu + 4I \rightleftharpoons 2CuI + I_2$$

Copper Carbonate Reagent. Dissolve in hot distilled water in a 1-liter Pyrex flask 250 g. of K_2CO_3 and add slowly 100 g. of finely pulverized $KHCO_3$, then add with shaking 15 g. of $CuSO_4 \cdot 5H_2O$ in 10 to 150 ml. of water, cool to 15°, make up to the mark with water, and filter.

PROCESS. *Copper Reduction.* Place in a 100-ml. volumetric flask 50 ml. of *copper carbonate reagent,* 20 ml. of the sugar solution, and heat to 48.5 to 49°. Stopper the flask and let stand 2.5 hours in a water bath at 48.5 to 49°. Remove from the bath, cool to 15°, add water to the mark, mix well, and, after the copper oxide has precipitated, filter on a dry paper, rejecting the first 20 to 25 ml.

Thiosulfate Titration. Acidify with *25% sulfuric acid* an aliquot of 50 ml. in a 250-ml. Erlenmeyer flask, the end-point being recognized by the change of color of the solution and no further evolution of carbon dioxide, add 10 ml. of *10% potassium iodide solution,* and determine the liberated iodine by titration with *standard 0.1 N sodium thiosulfate solution,* using *starch solution indicator.*

Blank. Carry out a blank determination with 20 ml. of water.

CALCULATION. Calculate the milligrams of levulose (L) in 20 ml. of the solution by the following formula:

$$L = \frac{(B - A) + 0.7}{0.26}$$

in which B is the number of milliliters of thiosulfate used in the blank determination and A the number used in the actual analysis.

DEXTROSE AND SUCROSE

Benedict and Osterberg Picrate–Picric Colorimetric Method.[69] The original method was designed for urine analysis.

Thomas and Dutcher Modification.[70] The modification (Pennsylvania State College Agricultural Experiment Station) is suited for the determination of sugars in plant extracts.

APPARATUS. *Colorimeter.*

REAGENTS. *Ethanol,* purified. Distil with KOH to remove acids and aldehydes and add 0.2 g. of $CaCO_3$ to prevent hydrolyzation by native acids.

Mercuric Nitrate Solution. Add to 80 ml. of HNO_3 with stirring 110 g. of $Hg(NO_3)_2 \cdot H_2O$, heat to boiling, and cool, then add 30 ml. of 5% NaOH solution, dilute to 1 liter, and store in the dark.

Picrate-Picric Reagent. Add to 500 ml. of 1% NaOH solution 36 g. of picric acid dried at 60° and purified if necessary.[71] Dilute with 400 ml. of hot water, shake until the picric acid has dissolved, cool, and dilute to 1 liter.

PROCESS. *Extraction.* Plunge 50 g. of the plant parts into boiling *purified ethanol.* Bring the ethanol extract to 70 to 75% by the addition of water if necessary. When convenient, filter, wash the material with *75% ethanol,* and dry at 70°, then grind to pass an 80-mesh sieve. Remove the remainder of the sugars by extracting the ground material in a continuous extractor with the ethanol filtrate. Transfer the colorless extract to a 250- or 500-ml. volumetric flask so as to secure a concentration of 0.1 to 1%.

Mercuric Nitrite Treatment. Evaporate at a low temperature a 20- to 100-ml. aliquot (0.025 to 0.150 g. of sugars), dissolve the residue in water, and wash into a 400-ml. beaker with 100 ml. of water. Add a slight excess of *mercuric nitrate solution* (usually 10 ml.), then gradually with stirring *solid sodium bicarbonate* until frothing ceases and the solution is alkaline to *litmus*, avoiding a large excess. Filter rapidly into a 250-ml. volumetric flask, wash with a little *5% sodium bicarbonate solution,* dilute the combined filtrate and washings to the mark, and shake.

Removal of Mercury. Pipet 30 to 50 ml. of the solution (0.01 to 0.07% of sugars) into a 75-ml. test tube, add 0.3 to 0.5 g. of *zinc dust*, followed by 1 drop of *hydrochloric acid* to reduce the mercury resulting from the formation of basic carbonate, taking care that sufficient sodium bicarbonate is present to prevent an acid reaction. Stopper loosely, shake, and after 15 minutes filter through a hardened paper. Test a portion of the filtrate to show the absence of mercury.

Color Formation. A. Reducing Sugars. Pipet 5 to 10 ml. of the filtrate into a 50-ml. Pyrex test tube, add 10 ml. of *picrate-picric reagent*, followed by 2 ml. of *25% sodium carbonate solution.* If less than 10 ml. of sugar solution was used, dilute to 22 ml. Prepare a color standard, using 10 ml. of *standard 0.025% dextrose solution*, 10 ml. of *picrate-picric reagent*, and 2 ml. of the *25% sodium carbonate solution.* Plug the tubes with cotton and immerse for 20 minutes in a water bath at 95°, then cool to room temperature.

Color Comparison. Dilute the solutions to 35 or 70 ml. according to the sugar present and compare in a colorimeter.

B. Sucrose. Invert a portion of the solution as directed in Part II, E2, and proceed as above to determine the color due to reducing sugar plus sucrose. Obtain the latter by difference.

Dextrose

Allihn Copper Reduction Gravimetric Method.[72] If dextrose is the only sugar present, whether in a natural juice or extract or in a solution formed by hydrolysis in starch determination, the Allihn method is both accurate and convenient.

Reagent. *Modified Fehling Reagent.* (1) *Copper Sulfate Solution.* Dissolve 34.639 g. of $CuSO_4 \cdot 5H_2O$ in water and dilute to 500 ml. (2) *Alkaline Tartrate Solution.* Dissolve 173 g. of Rochelle salt and 125 g. of KOH in water and dilute to 500 ml.

Process. *Copper Reduction.* Mix 30 ml. each of *solutions 1 and 2* with 60 ml. of water in a beaker and heat to boiling. While boiling, add from a pipet 25 ml. of the solution of the unknown, containing less than 0.250 g. of dextrose, clarified as directed in Part II, E3, if necessary, cover, and continue the boiling exactly 2 minutes. Filter immediately by decantation through a tared Gooch crucible containing a rather thick mat of acid-treated asbestos, transfer the cuprous oxide to the crucible, and wash well with hot water, then once with *ethanol* and once with *ether*.

Treatment of the Precipitate. If the precipitate of brick-red copper suboxide is believed to be pure as indicated by the color, dry at 100° to constant weight. If not pure, and in any event as a check, either reduce to metallic copper by heating the crucible within a Rose crucible in a stream of *hydrogen* and cooling in the same gas or, more conveniently, oxidize to cupric oxide at a dull red heat.

Calculation. Obtain the weight of dextrose from the weight of copper or cuprous oxide from the Allihn table herewith. If weighed as cupric oxide, convert to metallic copper by the factor 0.7986.

Romijn Iodometric Method. An oxidation reaction, employing iodine in weakly alkaline solutions, was shown by Romijn to be applicable to the determination of various aldehydes, especially formaldehyde[73] and dextrose.[74] The oxidation product of dextrose is sodium gluconate. Levulose and sucrose are not appreciably acted on, provided the proper degree of alkalinity, proportion of iodine to dextrose, and time of action are regulated. The reaction with dextrose is

$$CH_2OH(CHOH)_4CHO + 2I \rightarrow CH_2OH(CHOH)_4COONa + 2NaI + 2H_2O$$

The alkaline buffer used by Romijn was *borax*.

I. Dekker Modification.[75] This has proved of value in sugar laboratories of the East Indies and adjacent regions and has been employed by Yosida [76] for the determination of dextrose in the presence of levulose.

REAGENT. *Alkaline Buffer.* Dissolve 14.7 g. of $NaHCO_3$ and 2.95 g. of Na_2CO_3 in water and make up to 1 liter.

PROCESS. *Solution.* Place 25 ml. of a water solution or extract of the sample, containing about 30 mg. of dextrose, in a glass-stoppered bottle or Erlenmeyer flask.

Oxidation. Add *standard 0.1 N iodine solution* equivalent to ⅓ to ½ the dextrose present (usually 10 to 15 ml.), then 100 ml. of the *alkaline buffer solution.* Moisten the stopper to form a trap to prevent the escape of iodine, replace in the bottle or flask, and keep in a dark place for 40 minutes.

Titration. Add 12 to 15 ml. of *25% sulfuric acid* and titrate with *standard 0.1 N sodium thiosulfate solution.*

Blank. Run a blank using 25 ml. of water and the same amount of reagents as in the actual analysis and introduce the correction.

CALCULATION. Use: 1 ml. of standard 0.1 *N* iodine solution = 9.005 mg. of dextrose.

II. Willstätter and Schudel Modification.[77] In this modification, as in that of Voorhies and Alvarado,[78] only a slight excess of sodium hydroxide is used. The method has found favor with Judd,[79] Baker and Hulton,[80] and others.

The solution containing dextrose is mixed with 1.5 to 4 times the volume of *iodine solution* required by the Romijn reaction, then 1.5 volumes of *0.1 N sodium hydroxide solution* are added dropwise with shaking. After being allowed to stand 1.5 to 5 minutes (for small amounts of dextrose 20 minutes), *dilute sulfuric acid* is added to faint reaction and the excess of iodine is titrated back with *sodium thiosulfate solution.*

III. Other Modifications. Auerbach and Bödlander [81] found 100 ml. of a solution of 0.2 moles each of *sodium carbonate* and *bicarbonate* in 1 liter adequate for 25 ml. of a solution containing not more than 100 mg. of dextrose. Dekker, as given above, further adjusted the proportion of the carbonate to the volume of liquid and the dextrose content and showed that an increase in the amount of iodine over that given by Auerbach and Bödlander is essential.

Noyons [82] dilutes to 10 ml. the solution containing 0.2 to 2.0 mg. of dextrose, adds 1 ml. of *0.1 N iodine solution,* shakes, then after allowing to stand 1 minute, adds 1 ml. of *0.2 N sodium hydroxide solution,* and, finally, after letting it stand for a time, adds 6 ml. of *0.25% hydrochloric acid* and titrates back with *standard sodium thiosulfate solution.*

Still other modifications, differing chiefly in the alkaline constituents and their proportions, are noted by Nichols.[83]

Scales Simplified Cuprous Chloride Iodometric Method.[84] The notable features of this method are the formation of copper chloride from the sulfate as in the original Scales method [85] and the liberation of carbon dioxide from the carbonate in the Benedict-Clark alkaline copper sulfate solution [86] which, as first observed by Cammidge,[87] prevents oxidation of the cuprous chloride in the reduction flask. The method may be used for reducing sugars other than dextrose, provided the milligrams of the pure sugar corresponding to each milliliter of thiosulfate is determined by actual analysis.

REAGENTS. *Benedict-Clark Alkaline Copper Sulfate Reagent.* Dissolve 16 g. of $CuSO_4 \cdot 5H_2O$ in 125 to 150 ml. of water and pour into a hot solution of 150 g. of $Na_3C_6H_5O_7 \cdot 2H_2O$, 130 g. of Na_2CO_3, and 10 g. of $NaHCO_3$ in 650 ml. of water, then make up to approximately 1 liter, completing the volume after cooling.

Standard Sodium Thiosulfate Solution, 0.04 *N.* Dissolve 10 g. of the pure salt in 1 liter of water. Standardize against 0.05 *N* $K_2Cr_2O_7$, 15 ml. of which should theoretically correspond to 18.75 ml. of the thiosulfate so-

DEXTROSE FROM COPPER AND CUPROUS OXIDE (ALLIHN)

Cu	Cu_2O	Dex-trose	Cu	Cu_2O	Dex-trose	Cu	Cu_2O	Dex-trose	Cu	Cu_2O	Dex-trose
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
11	12.4	6.6	71	79.9	36.3	131	147.5	66.7	191	215.0	97.8
12	13.5	7.1	72	81.1	36.8	132	148.6	67.2	192	216.2	98.4
13	14.6	7.6	73	82.2	37.3	133	149.7	67.7	193	217.3	98.9
14	15.8	8.1	74	83.3	37.8	134	150.9	68.2	194	218.4	99.4
15	16.9	8.6	75	84.4	38.3	135	152.0	68.8	195	219.5	100.0
16	18.0	9.0	76	85.6	38.8	136	153.1	69.3	196	220.7	100.5
17	19.1	9.5	77	86.7	39.3	137	154.2	69.8	197	221.8	101.0
18	20.3	10.0	78	87.8	39.8	138	155.4	70.3	198	222.9	101.5
19	21.4	10.5	79	88.9	40.3	139	156.5	70.8	199	224.0	102.0
20	22.5	11.0	80	90.1	40.8	140	157.6	71.3	200	225.2	102.6
21	23.6	11.5	81	91.2	41.3	141	158.7	71.8	201	226.3	103.1
22	24.8	12.0	82	92.3	41.8	142	159.9	72.3	202	227.4	103.7
23	25.9	12.5	83	93.4	42.3	143	161.0	72.9	203	228.5	104.2
24	27.0	13.0	84	94.6	42.8	144	162.1	73.4	204	229.7	104.7
25	28.1	13.5	85	95.7	43.4	145	163.2	73.9	205	230.8	105.3
26	29.3	14.0	86	96.8	43.9	146	164.4	74.4	206	231.9	105.8
27	30.4	14.5	87	97.9	44.4	147	165.5	74.9	207	233.0	106.3
28	31.5	15.0	88	99.1	44.9	148	166.6	75.5	208	234.2	106.8
29	32.7	15.5	89	100.2	45.4	149	167.7	76.0	209	235.3	107.4
30	33.8	16.0	90	101.3	45.9	150	168.9	76.5	210	236.4	107.9
31	34.9	16.5	91	102.4	46.4	151	170.0	77.0	211	237.6	108.4
32	36.0	17.0	92	103.6	46.9	152	171.1	77.5	212	238.7	109.0
33	37.2	17.5	93	104.7	47.4	153	172.3	78.1	213	239.8	109.5
34	38.3	18.0	94	105.8	47.9	154	173.4	78.6	214	240.9	110.0
35	39.4	18.5	95	107.0	48.4	155	174.5	79.1	215	242.1	110.6
36	40.5	18.9	96	108.1	48.9	156	175.6	79.6	216	243.2	111.1
37	41.7	19.4	97	109.2	49.4	157	176.8	80.1	217	244.3	111.6
38	42.8	19.9	98	110.3	49.9	158	177.9	80.7	218	245.4	112.1
39	43.9	20.4	99	111.5	50.4	159	179.0	81.2	219	246.6	112.7
40	45.0	20.9	100	112.6	50.9	160	180.1	81.7	220	247.7	113.2
41	46.2	21.4	101	113.7	51.4	161	181.3	82.2	221	248.7	113.7
42	47.3	21.9	102	114.8	51.9	162	182.4	82.7	222	249.9	114.3
43	48.4	22.4	103	116.0	52.4	163	183.5	83.3	223	251.0	114.8
44	49.5	22.9	104	117.1	52.9	164	184.6	83.8	224	252.4	115.3
45	50.7	23.4	105	118.2	53.5	165	185.8	84.3	225	253.3	115.9
46	51.8	23.9	106	119.3	54.0	166	186.9	84.8	226	254.4	116.4
47	52.9	24.4	107	120.5	54.5	167	188.0	85.3	227	255.6	116.9
48	54.0	24.9	108	121.6	55.0	168	189.1	85.9	228	256.7	117.4
49	55.2	25.4	109	122.7	55.5	169	190.3	86.4	229	257.8	118.0
50	56.3	25.9	110	123.8	56.0	170	191.4	86.9	230	258.9	118.5
51	57.4	26.4	111	125.0	56.5	171	192.5	87.4	231	260.1	119.0
52	58.5	26.9	112	126.1	57.0	172	193.6	87.9	232	261.2	119.6
53	59.7	27.4	113	127.2	57.5	173	194.8	88.5	233	262.3	120.1
54	60.8	27.9	114	128.3	58.0	174	195.9	89.0	234	263.4	120.7
55	61.9	28.4	115	129.6	58.6	175	197.0	89.5	235	264.6	121.2
56	63.0	28.8	116	130.6	59.1	176	198.1	90.0	236	265.7	121.7
57	64.2	29.3	117	131.7	59.6	177	199.3	90.5	237	266.8	122.3
58	65.3	29.8	118	132.8	60.1	178	200.4	91.1	238	268.0	122.8
59	66.4	30.3	119	134.0	60.6	179	201.5	91.6	239	269.1	123.4
60	67.6	30.8	120	135.1	61.1	180	202.6	92.1	240	270.2	123.9
61	68.7	31.3	121	136.2	61.6	181	203.8	92.6	241	271.3	124.4
62	69.8	31.8	122	137.4	62.1	182	204.9	93.1	242	272.5	125.0
63	70.9	32.3	123	138.5	62.6	183	206.0	93.7	243	273.6	125.5
64	72.1	32.8	124	139.6	63.1	184	207.1	94.2	244	274.7	126.0
65	73.2	33.3	125	140.7	63.7	185	208.3	94.7	245	275.8	126.6
66	74.3	33.8	126	141.9	64.2	186	209.4	95.2	246	277.0	127.1
67	75.4	34.3	127	143.0	64.7	187	210.5	95.7	247	278.1	127.6
68	76.6	34.8	128	144.1	65.2	188	211.7	96.3	248	279.2	128.1
69	77.7	35.3	129	145.2	65.7	189	212.8	96.8	249	280.3	128.7
70	78.8	35.8	130	146.4	66.2	190	213.9	97.3	250	281.5	129.2

DEXTROSE FROM COPPER AND CUPROUS OXIDE (ALLIHN)—*Concluded*

Cu	Cu_2O	Dextrose	Cu	Cu_2O	Dextrose	Cu	Cu_2O	Dextrose	Cu	Cu_2O	Dextrose
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
251	282.6	129.7	306	344.5	159.8	361	406.4	190.6	416	468.4	222.2
252	283.7	130.3	307	345.6	160.4	362	407.6	191.1	417	469.5	222.8
253	284.8	130.8	308	346.8	160.9	363	408.7	191.7	418	470.6	223.3
254	286.0	131.4	309	347.9	161.5	364	409.8	192.3	419	471.8	223.9
255	287.1	131.9	310	349.0	162.0	365	410.9	192.9	420	472.9	224.5
256	288.2	132.4	311	350.1	162.6	366	412.1	193.4	421	474.0	225.1
257	289.3	133.0	312	351.3	163.1	367	413.2	194.0	422	475.6	225.7
258	290.5	133.5	313	352.4	163.7	368	414.3	194.6	423	476.2	226.3
259	291.6	134.1	314	353.5	164.2	369	415.4	195.1	424	477.4	226.9
260	292.7	134.6	315	354.6	164.8	370	416.6	195.7	425	478.5	227.5
261	293.8	135.1	316	355.8	165.3	371	417.7	196.3	426	479.6	228.0
262	295.0	135.7	317	356.9	165.9	372	418.8	196.8	427	480.7	228.6
263	296.1	136.2	318	358.0	166.4	373	420.0	197.4	428	481.9	229.2
264	297.2	136.8	319	359.1	167.0	374	421.1	198.0	429	483.0	229.8
265	298.3	137.3	320	360.3	167.5	375	422.2	198.6	430	484.1	230.4
266	299.5	137.8	321	361.4	168.1	376	423.3	199.1	431	485.3	231.0
267	300.6	138.4	322	362.5	168.6	377	424.5	199.7	432	486.4	231.6
268	301.7	138.9	323	363.7	169.2	378	425.6	200.3	433	487.5	232.2
269	302.8	139.5	324	364.8	169.7	379	426.7	200.8	434	488.6	232.8
270	304.0	140.0	325	365.9	170.3	380	427.8	201.4	435	489.7	233.4
271	305.1	140.6	326	367.0	170.9	381	429.0	202.0	436	490.9	233.9
272	306.2	141.1	327	368.2	171.4	382	430.1	202.5	437	492.0	234.5
273	307.3	141.7	328	369.3	172.0	383	431.2	203.1	438	493.1	235.1
274	308.5	142.2	329	370.4	172.5	384	432.3	203.7	439	494.3	235.7
275	309.6	142.8	330	371.5	173.1	385	433.5	204.3	440	495.4	236.3
276	310.7	143.3	331	372.7	173.7	386	434.6	204.8	441	496.5	236.9
277	311.9	143.9	332	373.8	174.2	387	435.7	205.4	442	497.6	237.5
278	313.0	144.4	333	374.9	174.8	388	436.8	206.0	443	498.8	238.1
279	314.1	145.0	334	376.0	175.3	389	438.0	206.5	444	499.9	238.7
280	315.2	145.5	335	377.2	175.9	390	439.1	207.1	445	501.0	239.3
281	316.4	146.1	336	378.3	176.5	391	440.2	207.7	446	502.1	239.8
282	317.5	146.6	337	379.4	177.0	392	441.3	208.3	447	503.2	240.4
283	318.6	147.2	338	380.5	177.6	393	442.4	208.8	448	504.4	241.0
284	319.7	147.7	339	381.7	178.1	394	443.6	209.4	449	505.5	241.6
285	320.9	148.3	340	382.8	178.7	395	444.7	210.0	450	506.6	242.2
286	322.0	148.8	341	383.9	179.3	396	445.9	210.6	451	507.8	242.8
287	323.1	149.4	342	385.0	179.8	397	447.0	211.2	452	508.9	243.4
288	324.2	149.9	343	386.2	180.4	398	448.1	211.7	453	510.0	244.0
289	325.4	150.5	344	387.3	180.9	399	449.2	212.3	454	511.1	244.6
290	326.5	151.0	345	388.4	181.5	400	450.3	212.9	455	512.3	245.2
291	327.4	151.6	346	389.6	182.1	401	451.5	213.5	456	513.4	245.7
292	328.7	152.1	347	390.7	182.6	402	452.6	214.1	457	514.5	246.3
293	329.9	152.7	348	391.8	183.2	403	453.7	214.6	458	515.6	246.9
294	331.0	153.2	349	392.9	183.7	404	454.8	215.2	459	516.8	247.5
295	332.1	153.8	350	394.0	184.3	405	456.0	215.8	460	517.9	248.1
296	333.3	154.3	351	395.2	184.9	406	457.1	216.4	461	519.0	248.7
297	334.4	154.9	352	396.3	185.4	407	458.2	217.0	462	520.1	249.3
298	335.5	155.4	353	397.4	186.0	408	459.4	217.5	463	521.3	249.9
299	336.6	156.0	354	398.6	186.6	409	460.5	218.1			
300	337.8	156.5	355	399.7	187.2	410	461.6	218.7			
301	338.9	157.1	356	400.8	187.7	411	462.7	219.3			
302	340.0	157.6	357	401.9	188.3	412	463.8	219.9			
303	341.1	158.2	358	403.1	188.9	413	465.0	220.4			
304	342.3	158.7	359	404.2	189.4	414	466.1	221.0			
305	343.4	159.3	360	405.3	190.0	415	467.2	221.6			

Maltose from Copper and Cuprous Oxide (Wein)

Cu	Cu_2O	Maltose	Cu	Cu_2O	Maltose	Cu	Cu_2O	Maltose	Cu	Cu_2O	Maltose
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
31	34.9	26.1	76	85.6	65.4	121	136.2	105.3	166	186.9	145.8
32	36.0	27.0	77	86.7	66.2	122	137.4	106.2	167	188.0	146.7
33	37.2	27.9	78	87.8	67.1	123	138.5	107.1	168	189.1	147.6
34	38.3	28.7	79	88.9	68.0	124	139.6	108.0	169	190.3	148.5
35	39.4	29.6	80	90.1	68.9	125	140.7	108.9	170	191.4	149.4
36	40.5	30.5	81	91.2	69.7	126	141.9	109.8	171	192.5	150.3
37	41.7	31.3	82	92.3	70.6	127	143.0	110.7	172	193.6	151.2
38	42.8	32.2	83	93.4	71.5	128	144.1	111.6	173	194.8	152.0
39	43.9	33.1	84	94.6	72.4	129	145.2	112.5	174	195.9	152.9
40	45.0	33.9	85	95.7	73.2	130	146.4	113.4	175	197.0	153.8
41	46.2	34.8	86	96.8	74.1	131	147.5	114.3	176	198.1	154.7
42	47.3	35.7	87	97.9	75.0	132	148.6	115.2	177	199.3	155.6
43	48.4	36.5	88	99.1	75.9	133	149.7	116.1	178	200.4	156.5
44	49.5	37.4	89	100.2	76.8	134	150.9	117.0	179	201.5	157.4
5	50.7	38.3	90	101.3	77.7	135	152.0	117.9	180	202.6	158.3
46	51.8	39.1	91	102.4	78.6	136	153.1	118.8	181	203.8	159.2
47	52.9	40.0	92	103.6	79.5	137	154.2	119.7	182	204.9	160.1
48	54.0	40.9	93	104.7	80.3	138	155.4	120.6	183	206.0	160.9
49	55.2	41.8	94	105.8	81.2	139	156.5	121.5	184	207.1	161.8
50	56.3	42.6	95	107.0	82.1	140	157.6	122.4	185	208.3	162.7
51	57.4	43.5	96	108.1	83.0	141	158.7	123.3	186	209.4	163.6
52	58.5	44.4	97	109.2	83.9	142	159.9	124.2	187	210.5	164.5
53	59.7	45.2	98	110.3	84.8	143	161.0	125.1	188	211.7	165.4
54	60.8	46.1	99	111.5	85.7	144	162.1	126.0	189	212.8	166.3
55	61.9	47.0	100	112.6	86.6	145	163.2	126.9	190	213.9	167.2
56	63.0	47.8	101	113.7	87.5	146	164.4	127.8	191	215.0	168.1
57	64.2	48.7	102	114.8	88.4	147	165.5	128.7	192	216.2	169.0
58	65.3	49.6	103	116.0	89.2	148	166.6	129.6	193	217.3	169.8
59	66.4	50.4	104	117.1	90.1	149	167.7	130.5	194	218.4	170.7
60	67.6	51.3	105	118.2	91.0	150	168.9	131.4	195	219.5	171.6
61	68.7	52.2	106	119.3	91.9	151	170.0	132.3	196	220.7	172.5
62	69.8	53.1	107	120.5	92.8	152	171.1	133.2	197	221.8	173.4
63	70.9	53.9	108	121.6	93.7	153	172.3	134.1	198	222.9	174.3
64	72.1	54.8	109	122.7	94.6	154	173.4	135.0	199	224.0	175.2
65	73.2	55.7	110	123.8	95.5	155	174.5	135.9	200	225.2	176.1
66	74.3	56.6	111	125.0	96.4	156	175.6	136.8	201	226.3	177.0
67	75.4	57.4	112	126.1	97.3	157	176.8	137.7	202	227.4	177.9
68	76.6	58.3	113	127.2	98.1	158	177.9	138.6	203	228.5	178.7
69	77.7	59.2	114	128.3	99.0	159	179.0	139.5	204	229.7	179.6
70	78.8	60.1	115	129.6	99.9	160	180.1	140.4	205	230.8	180.5
71	79.9	61.0	116	130.6	100.8	161	181.3	141.3	206	231.9	181.4
72	81.1	61.8	117	131.7	101.7	162	182.4	142.2	207	233.0	182.3
73	82.2	62.7	118	132.8	102.6	163	183.5	143.1	208	234.2	183.2
74	83.3	63.6	119	134.0	103.5	164	184.6	144.0	209	235.3	184.1
75	84.4	64.5	120	135.1	104.4	165	185.8	144.9	210	236.4	185.0

MALTOSE FROM COPPER AND CUPROUS OXIDE (WEIN)—*Concluded*

Cu	Cu_2O	Maltose	Cu	Cu_2O	Maltose	Cu	Cu_2O	Maltose	Cu	Cu_2O	Maltose
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
211	237.6	185.9	236	265.7	208.3	261	293.8	230.7	286	322.0	253.1
212	238.7	186.8	237	266.8	209.1	262	295.0	231.6	287	323.1	254.0
213	239.8	187.7	238	268.0	210.0	263	296.1	232.5	288	324.2	254.9
214	240.9	188.6	239	269.1	210.9	264	297.2	233.4	289	325.4	255.8
215	242.1	189.5	240	270.2	211.8	265	298.3	234.3	290	326.5	256.6
216	243.2	190.4	241	271.3	212.7	266	299.5	235.2	291	327.4	257.5
217	244.3	191.2	242	272.5	213.6	267	300.6	236.1	292	328.7	258.4
218	245.4	192.1	243	273.6	214.5	268	301.7	237.0	293	329.9	259.3
219	246.6	193.0	244	274.7	215.4	269	302.8	237.9	294	331.0	260.2
220	247.7	193.9	245	275.8	216.3	270	304.0	238.8	295	332.1	261.1
221	248.7	194.8	246	277.0	217.2	271	305.1	239.7	296	333.2	262.0
222	249.9	195.7	247	278.1	218.1	272	306.2	240.6	297	334.4	262.8
223	251.0	196.6	248	279.2	219.0	273	307.3	241.5	298	335.5	263.7
224	252.4	197.5	249	280.3	219.9	274	308.5	242.4	299	336.6	264.6
225	253.3	198.4	250	281.5	220.8	275	309.6	243.3	300	337.8	265.5
226	254.4	199.3	251	282.6	221.7	276	310.7	244.2			
227	255.6	200.2	252	283.7	222.6	277	311.9	245.1			
228	256.7	201.1	253	284.8	223.5	278	313.0	246.0			
229	257.8	202.0	254	286.0	224.4	279	314.1	246.9			
230	258.9	202.9	255	287.1	225.3	280	315.2	247.8			
231	260.1	203.8	256	288.2	226.2	281	316.4	248.7			
232	261.2	204.7	257	289.3	227.1	282	317.5	249.6			
233	262.3	205.6	258	290.5	228.0	283	318.6	250.4			
234	263.4	206.5	259	291.6	228.9	284	319.7	251.3			
235	264.6	207.4	260	292.7	229.8	285	320.9	252.2			

lution. Standardize also by making determinations on pure dextrose supplied by the U. S. Bureau of Standards.

PROCESS. *Copper Reduction.* Introduce 20 ml. of the *Benedict-Clark alkaline copper sulfate reagent* into a 300-ml. Erlenmeyer flask, then add 10 ml. of the sugar solution containing not more than 20 mg. of dextrose. If more than 20 mg. of dextrose is present in the 10 ml., use 35 ml. of the reagent. Heat the mixture over a flame or hot plate. If the latter, close the flask with a two-hole rubber stopper to hasten the heating, thus attaining the boiling point in about 5 and 6.75 minutes for a total volume of 25 and 35 ml. respectively. If a free flame is used, adjust the flow of gas so that the boiling point is reached in about 1 minute less than over the hot plate. Continue the boiling exactly 3 minutes, then cool as quickly as possible under a stream of water.

Iodine Absorption. Add to the cooled mixture 100 ml. of *2.4% acetic acid* and sufficient *standard 0.04 N iodine solution* to leave an excess of 5 to 8 ml., then introduce 25 ml. of *2.5% hydrochloric acid* from a pipet in such a manner as to spread the acid over the sides of the flask and shake vigorously. Wash the pipet and sides of the flask with a few milliliters of water and rotate the flask for exactly 1 minute, thus dissolving completely the precipitate.

Thiosulfate Titration. Run into the liquid *standard 0.04 N sodium thiosulfate solution,* while mixing by rotation, until the brown or blue solution changes in color to light sea green. Add 2 ml. of *starch solution* and continue the titration until the dark blue suddenly fades and the solution acquires the light blue color characteristic of an acidified copper solution.

Calculation. The dextrose value of each milliliter of thiosulfate solution is 1.15 for a sugar content of approximately 1.5 mg. and is 1.12 mg. for an average sugar content of from 3 to 24 mg.

Ofner Modification.[88] This modification, claimed to be more reliable than the Herzfeld method, differs from the Scales method chiefly in that the *alkaline copper reagent* contains *disodium phosphate* and *Rochelle salt* in place of sodium citrate. Doubtless numberless salts in various proportions would serve the same purpose.

Note. Ionesco-Matiu [89] heats a measured volume of *standard ferricyanide solution* to boiling and tests the sugar solution until reduction to ferrocyanide is complete, as shown by the cherry-red color with *picric acid.* One molecule of ferricyanide oxidizes one molecule of dextrose.

Maltose

Wein Copper Reduction Gravimetric Method.[90] Reagents. *Fehling-Soxhlet Solutions 1 and 2.* See Reducing Sugars above.

Process. *Copper Reduction.* Measure into a beaker a mixture of 25 ml. each of *Fehling-Soxhlet solutions 1 and 2,* add 25 ml. of the solution of the sample containing not more than 1% of maltose, heat to boiling, continue the boiling for exactly 4 minutes, and filter at once through asbestos. Dry at 100° and weigh as cuprous oxide or reduce in *hydrogen* and weigh as metallic copper as described for the Allihn Method above.

Calculation. In the table published by Wein, only the weights of metallic copper and maltose appear; in the table above the weights of cuprous oxide are also given.

Note. Brown, Morris, and Millar [91] and also Ling and Baker [92] have found that the results, using Wein's table, are about 5% low. Zäch [93] proposes a somewhat different method and a new table. See Browne and Zerban: *Chemical and Physical Methods of Sugar Analysis,* New York, 3rd Ed., 1941, p. 797.

Lactose

See Part II, G1.

Starch

See also Part II, A2, I3, and J1.

Starch Content of Foods. Except in polar regions, starch is the chief calorific constituent of the human and herbivorous animal dietary. It exceeds in percentage amount the sum of all other nutrients in the cereal grains, dried peas and beans, potatoes, sweet potatoes, yams, tapioca, and similar starchy subterranean foods. The bread- and rice-eating nations depend alike on starch for warmth and muscular energy. On the average, over two-thirds of wheat, rye, and corn (maize) kernels and over three-fourths of white flour and polished rice is starch. Potatoes, reduced to the same water content, contain starch in similar high proportion. Black and white pepper and ginger among spices are rich in starch.

It is remarkable that, although small amounts—often mere traces—of starch occur in many foods, those containing intermediate amounts are unusual. Nature seems averse to an equal division of the nutrients into starch, protein, and oil. Oil seeds are generally starch-free, the starch being replaced partly by oil and partly by protein; the peanut, containing about 5% of starch and the cocoa bean less than 10%, are exceptions.

Formation and Transformation of Starch. Many fruits, when green, contain starch that passes into sugar at full ripeness; the banana is a notable example. On the other hand, sugars present in very early stages of development of vegetable foods may be converted into starch at later stages. The reversible reactions may be expressed thus:

$$\text{Starch} \rightleftharpoons \text{reducing sugars} \rightleftharpoons \text{sucrose}$$

Whatever the transformation from starch to sugars and sugars to starch, the original source is in the leaf and other green parts of the plant organs functioning as leaves. It is in the leaf that carbon dioxide of the air combining with water drawn up through the roots and stem forms starch as the first visible product of photosynthesis. This most remarkable of all biological reactions, in which the chlorophyl grain plays a mysterious role, may be expressed as follows:

$$6CO_2 + 5H_2O \longrightarrow C_6H_{10}O_5 + 12O \uparrow$$

This early formed carbohydrate goes through various metamorphoses and enters into the formation of proteins, fats, acids, cellulose, and numerous other organic compounds. All the organic substances of foods are end-products of a series of reactions starting with the simple one that takes place in the leaf. In the animal body the series of complicated reactions ending in the exhalation of carbon dioxide are in substance the reverse of photosynthesis—the burning of starch or its equivalent, thus returning to the air the carbon dioxide originally taken up by the leaves of plants functioning in the sunlight.

Microscopic Structure. See Part I, A.

Determination of Starch. *Application.* Starch determination is essential in physiological experiments wherein the formation or disappearance of starch in leaves, stems, roots, fruits, and seeds or the rate of digestion of starch in the animal organ is significant.

Certain industries, notably the manufacture of commercial starch and products made therefrom (glucose, starch sugar, dextrin, etc.), the manufacture of malt liquors, also alcohol, distilled liquors, and vinegar, so far as these are made from starchy seeds, roots, and tubers, as distinguished from saccharine products such as cane or beet sugar, molasses, and fruit juices, are dependent on the starch content of the raw material. The value of the raw material, other things being equal, is proportional to the starch content. The completeness of removal in manufacture is indicated by the starch content of the residue. The percentage of starch is often a measure of purity or, on the other hand, of adulteration with starchy material.

The source of starch may often be learned by microscopic examination. See Part I, A.

Sachsse Direct Hydrolysis Copper Reduction Gravimetric Method.[94] PROCESS. *Removal of Oil and Fat.* Weigh 4 g. of the sample into a filter that will retain the smallest starch grains. If the material is an oil seed or contains a considerable amount of fat, fatty oil, essential oil, or other ether-soluble matter, extract with *ether* (not necessarily anhydrous) until practically all the ether-soluble matter has been removed. This treatment is unnecessary if the product is commercial starch, tapioca, white flour, bread, dried potatoes, and other substances containing only small amounts of ether-soluble material.

Removal of Soluble Reducing Substances. Allow the ether of the extracted residue to evaporate and wash with 150 ml. of *10% ethanol* or until all the sugar and other soluble reducing matters, such as dextrins and tannins, are removed. The washing may be performed with cold water alone, provided no starch passes through the paper, but dilute ethanol, as recommended by Lindsey, often yields a clearer filtrate.

Hydrolysis. Carefully wash the residue into a 500-ml. volumetric flask with 200 ml.

of water contained in a small wash-bottle, gently rubbing the paper with the tip of the little finger to loosen any adhering starch. Add 20 ml. of *hydrochloric acid* (sp.gr. 1.125) and heat for 3 hours on a boiling water bath to convert the starch into dextrose. Cool, nearly neutralize with *sodium hydroxide solution*, dilute to the mark, shake, and filter through a dry paper.

Copper Reduction. In an aliquot of the solution determine dextrose by the Allihn, the Munson and Walker, or other Copper Reduction method (above).

CALCULATION. Multiply the weight of dextrose by 0.9 to obtain the corresponding weight of starch. Express the result as *starch* or *pure starch* if the fiber or insoluble tannin compounds present in the sample are inconsiderable, otherwise as *crude starch* or *copper-reducing matter by direct hydrolysis calculated as starch.*

NOTE. This method yields accurate results on commercial starch and tapioca, also on white flour and other products containing small amounts of fiber, sugar and other soluble reducing substances which have been removed by washing with water or dilute ethanol. In fact, the results are more accurate than by the diastase method, since complete removal of starch by the enzyme from minute lumps is difficult. When, however, the sample, such as oats, barley, and bran, is rich in fiber, or, as is true of cloves and allspice, contains tannin matter insoluble in water, the results are at best only approximate. If microscopic examination of the sample shows no starch, the figures obtained represent copper-reducing substances other than starch.

Märcker Diastase Copper Reduction Gravimetric Method.[95] REAGENT. *Malt Extract.* Powder 100 g. of freshly prepared brewer's malt, digest immediately before using with 1 liter of water for 2 to 3 hours, and filter. Taka-diastase, the product of a Japanese fungus, has the advantage of being free from reducing matter, but in the experience of the writer and others is not a satisfactory substitute for barley malt.

PROCESS. Grind a portion of the sample to an impalpable powder so that at least it passes a sieve with holes 0.5 mm. in diameter. Extract with *ether* and *10% ethanol* as directed above for the Sachsse method.

Conversion to Starch Paste. Transfer the residue to a small mortar by means of a jet of water, taking care to loosen any starchy matter adhering to the paper. Decant the supernatant liquid into a beaker and thoroughly grind the residue, repeating the operation until all the material has been collected in the beaker together with about 100 ml. of liquid. Heat cautiously to boiling over thin asbestos board with constant stirring, taking care that none of the material sticks to the glass, boil gently for 30 minutes, continuing the stirring, and replace the water lost by evaporation. These precautions in the early stages of the process must be strictly observed.

Diastase Digestion. Place the beaker and its contents of thin starch paste in a water bath at 55 to 60°, taking care that the water in the bath and in the beaker are at the same level. When the temperature becomes constant add from a pipet 10 ml. of *malt extract* and digest for 1 hour with occasional stirring. Remove from the bath and boil a second time for 15 minutes, cool, and digest with a new portion of 10 ml. of *malt extract.* Boil a third time, cool, and transfer to a 250-ml. volumetric flask, make up to the mark, shake, and filter through a dry paper; pipet 200 ml. into a 500-ml. volumetric flask.

Acid Hydrolysis. Add 20 ml. of *hydrochloric acid* (sp.gr. 1.125), heat on a boiling water bath for 3 hours, cool, nearly neutralize with *sodium hydroxide solution*, and make up to the mark.

Copper Reduction. In an aliquot determine dextrose as in the Sachsse method above.

Blank Determination. Carry along with one or more actual analyses two or three blank analyses as follows. Pipet 20 ml. of the malt extract into a 250-ml. flask, digest, and boil as directed above, but aliquot only once and without filtering. Determine the copper reduction, find in the table the corresponding weight of dextrose, divide by 6 to allow for the lesser dilution, and subtract from the weight of dextrose found in the actual analysis.

A common error is to correct the weight of copper rather than that of the dextrose, overlooking that the reducing power of the dextrose solution differs with the dextrose concentration, thus making a calculation table necessary.

CALCULATION. Multiply the corrected weight of dextrose by 0.9 to obtain the weight of starch (pure starch).

von Asbóth Barium Hydroxide Volumetric Method.[96] Zulkowsky prepared baryta starch, $(C_6H_{10}O_5)_4BaO$, by the action of barium hydroxide on soluble starch and von Asbóth found that the compound is formed quantitatively when starch paste is so treated. The method deserves further study.

PROCESS. *Preparation of Starch Solution.* Weigh 2 to 3 g. of the sample into a small mortar and rub up with small successive portions of boiling water (for flour, cold water), decanting after each into a 250-ml. volumetric flask until the starch is completely transferred, the final bulk being about 100 ml. Heat in a boiling water bath for 30 minutes, with occasional shaking.

Barium Hydroxide Precipitation. Cool, add 50 ml. of *standard 0.1 N barium hydroxide solution*, and shake for 2 minutes. Add *ethanol* to the mark and shake again, then allow to settle for 10 minutes. If the supernatant liquid is not clear, filter through glass wool contained in a tube or, better, asbestos in a Gooch crucible.

Titration. Pipet 50 ml. of the clear filtrate into a beaker and titrate the excess of barium hydroxide with *standard 0.1 N hydrochloric acid*, using *phenolphthalein indicator*. Titrate also 50 ml. of the *standard barium hydroxide solution* directly in the same manner.

CALCULATION. Multiply the difference between the two titrations by 5 and the product by 4.222, thus obtaining the weight of starch in the charge.

Lintner Polariscopic Method.[97] The procedure of Lintner (Munich) has been much used for approximate results. It depends on the formation by cold 10% hydrochloric acid of soluble starch with a specific rotation of +202.

APPARATUS. *Polariscope* with angular or sugar scale.

PROCESS. *Digestion.* Rub up in a mortar 2.5 g. of the finely powdered sample with 10 ml. of water and 20 ml. of *hydrochloric acid*, allow to stand 30 minutes, then pour into a 100-ml. volumetric flask, rinsing with *25% hydrochloric acid.*

Clarification. Add 5 ml. of *4% phosphotungstic acid solution*, make up to the mark with *25% hydrochloric acid*, mix, and filter.

Polarization. Without delay polarize in a 200-mm. tube.

CALCULATION. If the readings are in sugar degrees, convert into degrees of angular rotation. Obtain the percentage of starch (S) from the angular degrees (P) by the following formula:

$$\frac{100P \times 100}{2 \times 202 \times 2.5} = 9.901P$$

A formula for direct conversion for sugar degrees may be readily derived.

von Fellenberg Calcium Chloride-Iodine Precipitation Gravimetric Method.[98] Of the numerous methods devised in the search for a substitute for the tedious diastase method, a method embodying the solution of the starch in concentrated calcium chloride solutions seems the most promising. In common with the diastase method, grinding to an impalpable powder either dry or moist is essential

and the method is not applicable to oven or cooked products.

Process. *Extraction.* Moisten with water 0.3 to 1.0 g. of the flour or other starchy fat-free product ground to an impalpable powder, add 20 ml. of *50% calcium chloride solution*, and mix well. Heat on a boiling water bath for 30 minutes, then boil for 5 minutes, cool, make up to 100 ml., and filter first through cotton-wool and then through asbestos, centrifuging if necessary to secure a clear filtrate.

Iodine Precipitation. Add to 50 ml. of the filtrate *0.02 N iodine solution* until a flocculent precipitate is formed, avoiding a large excess. Allow to stand 24 hours, then add *asbestos* and filter through a Gooch crucible provided with an asbestos mat and wash 4 times with *5% calcium chloride solution* containing a few drops of *iodine solution*. Wash further with *60% ethanol* in such a manner that the precipitate is in contact with the wash ethanol for 5 minutes, then with *85% ethanol*, followed by 100 ml. of *90% ethanol*, supplemented by hot *95% ethanol* if the precipitate is not colorless. Complete the washing with *95% ethanol*, *5% calcium chloride*, and *absolute ether*. Dry the crucible and contents in a boiling water oven and weigh. Ignite and weigh again.

Calculation. The loss in weight represents the starch.

Mannich and Lenz Calcium Chloride Polarimetric Method.[99] This method combines the von Fellenberg procedure of dissolving the starch in calcium chloride solution and the Lintner procedure of polarizing the solution of soluble starch.

Hopkins Modification.[100] Hopkins (National Research Laboratories, Ottawa) found that neither the removal of soluble proteins by precipitation with zinc chloride solution nor their separation in another portion and deduction of their rotation, as employed in the Mannich and Lenz procedure, is feasible, but interfering substances, doubtless in considerable part sugars, may be removed by preliminary treatment with 73% ethanol.

Reagent. *Calcium Chloride Solution*, concentrated. Mix 200 g. of $CaCl_2 \cdot 6H_2O$ with 100 ml. of water, rendered faintly alkaline to phenolphthalein by NaOH solution.

Process. *Centrifuging.* Weigh 2.5 g. of the sample, ground to pass a 100-mesh sieve if not already an impalpable powder into a round-bottom 50-ml. lipped centrifuge tube, add 10 ml. of *73% ethanol*, and stir well with a glass rod, reserving the rod without cleaning for subsequent use. Centrifuge, decant, and repeat the stirring with 10 ml. of *65% ethanol* five times.

Calcium Chloride Extraction. Finally stir the residue with 10 ml. of water and pour into a 125-ml. Erlenmeyer flask, using 60 ml. of *concentrated calcium chloride solution* plus 2 ml. of *0.8% acetic acid* to rinse the tube. Heat to boiling and continue the boiling cautiously for 15 to 17 minutes, or preferably heat in a boiling water bath for 30 minutes,[101] taking care to bring particles adhering to the glass into the solution.

Polarization. Cool quickly in running water, make up to the mark in a 100-ml. volumetric flask with *concentrated calcium chloride solution*, shake, filter on a pleated paper, discarding the first 10 ml., and polarize in a 100-mm. tube. The average of two sets of ten readings should agree within 0.006 angular degrees.

Calculation. The following formula is applicable only to wheat flour or uncooked wheat products:

$$\frac{100 \times 100A}{200G} \qquad \frac{50A}{G}$$

in which S is the percentage of starch, A is the reading in angular degrees (or in degrees Ventzke converted into angular degrees), and G is the weight of the charge.

Notes. Etheredge[102] endorses the Mannich-Lenz procedure as modified by Hopkins, but substitutes washing on a filter with the

aid of slight suction for the washing by centrifuging.

The method, as tentatively adopted by the A.O.A.C. in 1944, permits: (1) filtering on paper using suction instead of centrifuging, (2) heating on asbestos over an Argand burner to diminish foaming, (3) use of Celite with Pyrex glass filters and Hirsch-type funnel with asbestos and suction, and (4) making 2 g. of the sample up to 100 ml. and polarizing in a 200-mm. tube (1° V. = 4.3225% of starch, 200 being assumed to be the specific rotation of all starches).

Sullivan Calcium Chloride Iodine-Iodide Copper Reduction Gravimetric Method.[103] In this method (Purdue University), the starch is dissolved in saturated calcium chloride solution and precipitated with iodine, as in the von Fellenberg method, then hydrolyzed to dextrose and the latter determined by copper reduction.

REAGENTS. *Calcium Chloride Solution*, saturated: Sp.gr. about 1.48, alkalinity about 0.025 *N*.

Iodine Reagent. Dissolve 2 g. of iodine and 6 g. of KI in 100 ml. of water.

Ammonium Sulfate Wash Solution. Mix 1 part of saturated $(NH_4)_2SO_4$ solution and 3 parts of water, then add a few drops of the iodine reagent.

PROCESS. *Calcium Chloride Treatment*. Weigh into a 250-ml. Pyrex beaker a quantity of the sample, ground to pass a 100-mesh sieve, containing about 0.1 g. of *starch*. Add a few milliliters of water, stir until completely wet, then add 40 to 50 ml. of *saturated calcium chloride solution*. If the alkalinity of the calcium chloride solution is less than 0.025 *N*, add to the charge 2 ml. of *0.1 N sodium hydroxide solution*. Heat to boiling and boil gently for 50 to 60 minutes, stirring occasionally and adding water to rinse down the side, and replace that lost by evaporation, maintaining a volume of about 60 ml. as indicated by a mark on the flask. Pour the mixture into a 100-ml. volumetric flask with a moderately wide neck, using a policeman and hot water to complete the transfer, cool to room temperature, dilute to the mark with water, mix, pour into a centrifuge tube, and whirl until the residue has settled. Remove any floating particles by straining through a coarse muslin cloth.

Ethanol Precipitation. Pipet an aliquot of 50 to 75 ml. of the clear solution into a 400-ml. beaker containing 2.5 volumes of *ethanol*, add 0.5 g. of *dry asbestos*, stir well, and let stand overnight. Filter on a Gooch crucible, wash at least 3 times with *75% ethanol*, and return the starch and asbestos to the beaker, rinsing with hot water, then dilute the mixture to 100 ml.

Iodine-Ammonium Sulfate Treatment. Heat the mixture just to boiling with continual stirring, cool to room temperature, and add 1 ml. of the *iodine reagent*. Stir well, add 50 ml. of cold *saturated ammonium sulfate solution*, and dilute to about 200 ml. Stir and allow the blue precipitate and the asbestos to settle beneath a clear amber liquid.

If the blue precipitate fails to settle, add more *iodine* and *ammonium sulfate solution*, stir, and let settle. After an hour collect the precipitate and asbestos on a Gooch crucible provided with a thin asbestos mat and wash several times with the *ammonium sulfate wash solution*, followed 3 times by *75% ethanol*. Transfer the precipitate (now partially decolorized) and asbestos back into the beaker with water, placing the crucible also in the beaker if the precipitate is not readily removed.

Acid Hydrolysis. Dilute the solution to a total volume of about 100 ml., add 15 ml. of *hydrochloric acid* (sp.gr. 1.1), and heat in a boiling water bath with stirring until the blue color has disappeared, then 30 minutes longer. Filter into a 500-ml. Kjeldahl flask through a sintered-glass crucible, add glass beads, and concentrate to about 100 ml., then reflux for 1 hour.

Copper Reduction. Nearly neutralize with

sodium hydroxide solution, transfer to a volumetric flask, remove an aliquot, and determine the amount of dextrose.

CALCULATION. Calculate the percentage of dextrose by the Munson and Walker, Allihn, or other standard method. Correct for the volume of insoluble matter and convert dextrose into starch by the factor 0.9.

Pucher and Vickery Calcium Chloride Iodometric Method.[104] APPARATUS. *Pulfrich Spectrophotometer.*

REAGENTS. *Calcium Chloride Solution,* 46%. Dissolve 216 g. of $CaCl_2$ in 250 ml. of water. Filter the hot solution through asbestos and keep in a warm place.

Ethanolic Sodium Hydroxide Solution, 0.25 *N.* Dilute 25 ml. of 5 *N* NaOH and 320 ml. of ethanol to 500 ml.

Iodine-Potassium Iodide Reagent. Dissolve 30 g. of iodine and 50 g. of KI in water and dilute to 250 ml.

Standard Potassium Iodate Solution, 0.01 *N.* Dissolve 0.1783 g. of KIO_3 in water and dilute to 500 ml.

Thymol Blue Indicator. A 0.04% solution.[105]

STANDARD CURVE. Determine in one or more samples of standard potato starch the moisture (in the experience of the writers best by drying at 120° in a stream of hydrogen) and ash, then obtain the pure anhydrous starch by difference.

Prepare standard solutions in hydrochloric acid and calcium chloride by heating equivalent portions of 0.05 g. of the dry *potato starch* as described below under Extraction, omitting the steps involving separation of extraneous constituents. To prepare the solution in calcium chloride, add 0.05 g. of *magnesium carbonate* and 5 ml. of water and heat in a boiling water bath for 15 minutes. Add 15 ml. of *46% calcium chloride solution,* a few drops of *caprylic alcohol,* and some angular *quartz pebbles,* then reflux for 2 hours. Dilute with 15 ml. of water, transfer to a 25 x 200 mm. test tube, dilute to 50 ml., and centrifuge. Carry aliquots containing 0.1 to 3.0 mg. of starch through the subsequent treatment as described below.

PROCESS. *Preparation.* Dry green tissues at 70 to 80° in an air current, grind to pass a 50- to 60-mesh sieve, and extract 2 to 5 g. with *75% ethanol* for at least 6 hours. Dry the residue, weigh, and grind to a powder.

Extraction. A. Rask Acid Procedure.[106] Weigh 0.2 g. of the ethanol extracted material into a heavy-walled 25 x 200 mm. Pyrex test tube, add 1.5 g. of *sharp sand,* 5 ml. of water, and a stout glass stirring rod, then heat in a boiling water bath for 15 minutes with occasional vigorous trituration with the rod. Cool to 10° and add slowly 5 ml. of *hydrochloric acid,* keeping the temperature below 22°. Grind with the rod for 30 seconds at 2-minute intervals for 10 minutes, holding the temperature at 20° between the intervals. Rinse the rod with a few drops of *Rask's acid* (21% hydrochloric acid) and centrifuge for 1 minute. Decant the supernatant liquid into a 25 x 200 mm. test tube with a 50-ml. mark and heat in a bath at 10 to 15°. Grind the centrifuged residue with the rod for 30 seconds, add 3 ml. of *Rask's acid,* grind as before for 5 to 7 minutes, centrifuge, and decant, repeating the treatment twice more, collecting the extract each time in the graduated test tube. Test the fourth extract with a drop of *0.01 N iodine solution;* if a definite blue color appears, make a fifth extraction. Finally wash the residue by grinding once more, using 10 ml. of water. Dilute the combined extracts to 50 ml. with water and hold in a refrigerator until the next step, which should be undertaken within 72 hours.

B. Calcium Chloride Procedure. Weigh 0.2 g. of the ethanol-extracted material into a test tube. Add 0.05 g. of *magnesium carbonate,* 1.5 g. of *sharp sand,* 5 ml. of water, and a stirring rod. Heat with occasional trituration in a boiling water bath for 15 minutes, then add 7 ml. of *46% calcium chloride solution,* previously heated in the same bath.

Grind while in the bath for 30 seconds at intervals for 10 minutes, rinse the rod with a few drops of water, centrifuge, and decant the extract into a 25 x 200 mm. test tube with a graduation mark at 50 ml. Grind for 30 seconds and return to the boiling water bath, then add 3 ml. of water and 5 ml. of hot *46% calcium chloride solution*. Repeat the grinding at intervals for 8 minutes and again centrifuge. Make two more extractions, test as directed above, after acidifying with *dilute hydrochloric acid*, and make a fifth extraction if necessary. Finally wash the residue by stirring with 5 ml. of water and centrifuge. Hold the combined extracts in a refrigerator.

Iodide Precipitation. Make a preliminary test on an aliquot of 0.2 ml. (acidified if calcium chloride was the solvent) with a drop of *0.01 N iodine solution*. If the color formed is light blue, pipet 10 ml. into a Gradwohl-Blavais sugar tube; if intense dark blue, pipet only 5 ml. or less. Dilute to 10 ml., add 2 drops of *thymol blue indicator*, and a slender rod with an enlarged end.

In the case of hydrochloric acid extract, cool the tube to 10° in a bath, add *10 N sodium hydroxide solution* dropwise with constant stirring to a faint blue color, then *2.3 N hydrochloric acid* to a yellow color and 1 ml. in excess, maintaining a temperature below 25°. If the aliquot is less than 5 ml., add 2 ml. of *20% sodium chloride solution* and precipitate the starch with 0.5 ml. of *46% calcium chloride solution* and 6 to 10 drops of *iodine-potassium iodide reagent*.

If the aliquot of the calcium chloride extract is smaller than 5 ml., add 1 ml. of *46% calcium chloride solution*, adjust the acidity as above, and add 2 ml. of *20% sodium chloride solution*, then precipitate the starch with 6 to 10 drops of *iodine-potassium iodide reagent*.

In either case (to the end) proceed as follows. Allow the tube to stand for 10 minutes, cover with a glass bulb and heat in a boiling water bath for 15 minutes. Cool to room temperature, break up any floating precipitate with a rod, and centrifuge. Draw off the supernatant liquid down to about 1 ml., through a long, thin capillary tube into a suction flask, ignoring floating particles of sublimed iodine. Add 2 ml. of *60% ethanol*, shake gently, centrifuge, draw off as before, and repeat the operation.

Starch Iodide Decomposition. Add 2 ml. of *0.25 N ethanolic sodium hydroxide solution* to the packed precipitate and triturate with a glass rod until nearly colorless. Wash the rod with *60% ethanol*. Heat in a bath at 70 to 80° for 5 minutes with gentle agitation to complete the decomposition. Cool, centrifuge, and decant the supernatant liquid, then wash the starch twice with 2 ml. of *60% ethanol*. Finally invert the tube and allow to drain thoroughly.

Starch Solution. To the starch precipitate add 5 ml. of water and heat with frequent shaking for 5 minutes in a boiling water bath. Cool, add 0.5 ml. of *2 N acetic acid*, mix well, and centrifuge. Decant into a 25 x 200 mm. test tube with marks at 20 and 50 ml. and immerse in a water bath at 15°, washing the residue by agitation with 3 ml. of water, and centrifuge.

Color Formation. To the starch solution add 2 ml. of *2 N acetic acid*, 0.5 ml. of *10% potassium iodide solution*, and 5 ml. of *0.01 N potassium iodate solution*, all exactly measured. Prepare simultaneously two compensating solutions containing 8 ml. of water, 2.5 ml. of *2 N acetic acid*, 0.5 ml. of *10% potassium iodide solution*, and 5 ml. of the *potassium iodate solution*, then dilute to 20 and 50 ml. respectively. Dilute the unknowns to 20 or 50 ml., depending on the color intensity, mix well, and allow to stand for 5 minutes.

Color Reading. Use 50-ml. dilution for over 0.5 mg. of starch and 20-ml. dilution for smaller amounts. Determine the extinction coefficient in the Pulfrich spectrophotometer,

using the compensating solution of the same volume on the control side, and obtain the equivalent of starch from the calibration curve.

CALCULATION. Refer the weight found to the 0.2-mg. charge and correct this in turn for the loss in weight during the preparation of the sample.

Woods Thiocyanate Gravimetric Method.[107] REAGENT. *Potassium Thiocyanate Solution, 40%.*

PROCESS. Heat at 50 to 60° for 2 hours with stirring 5 g. of the finely ground sample with 50 ml. of *40% potassium thiocyanate solution.* Centrifuge for 3 minutes and decant the clear solution. Precipitate the starch with double the volume of *ethanol,* centrifuge, wash with several portions of *ethanol,* and finally with a little cold water. Filter, dry, and weigh.

DEXTRIN

See Part II, E6 and F2.

GLYCOGEN

See Part II, H1.

b. ORGANIC ACIDS

FORMIC, ACETIC, PROPIONIC, AND BUTYRIC ACIDS

Osburn, Wood, and Werkman Partition Method.[108] The method is a modification of the Werkman method.[109] Its application has been in fermentation studies in plant control work and physiological investigations, conducted by the U. S. Department of Agriculture and the Iowa Agricultural Experiment Station. Lactic, pyruvic, and possibly other hydroxy or keto acids may be present, but the method applies only to the four acids named above. It is based on the distribution of the lower fatty acids between two immiscible solvents. Full details including calculation formulas are given for two-acid, three-acid, and four-acid mixtures.

Hillig and Knudsen Distillation Rate Volumetric Method.[110] The method involves fractional distillation under standard conditions, titration of 50- and 200-ml. fractions of the distillate, and computation by formulas involving tabulated data obtained with the pure acids.

APPARATUS. *Steam Distillation Assembly.*[111]

PROCESS. *Distillation.* Transfer a measured portion of the sample or a solution of the sample to the flask of the distillation assembly, add *50% sulfuric acid* until acid to *Congo paper*, and dilute to 150 ml. Distil at the rate of 200 ml. of distillate in 1 hour, as previously determined by trials. Collect one 50-ml. fraction, then one or more 200-ml. fractions according to the number of volatile acids present.

If the number of acids is known and formic acid is absent, collect as many fractions as there are acids, the first always being 50 ml. If formic acid is present, collect as many fractions as there are acids less one. In case the number of acids is unknown, collect one 50-ml. fraction, then three 200-ml. fractions.

Titration. Titrate each fraction with *0.05 N barium hydroxide solution*, using *phenolphthalein indicator;* also titrate 50- and 200-ml. fractions of blank distillates. Designate the corrected readings as T_1, T_2, T_3, and T_4.

Determination of Formic Acid. Determine the formic acid in the combined distillates, following the procedure described by Hillig and Clark.[112]

Distillation Rates. The distillation rates of five volatile acids, using the apparatus and following the directions of Hillig and Clark, is shown in the table below.

CALCULATION. For approximate results, follow the instruction of Hillig and Clark and base calculations on the figures given in the table below, but for greater accuracy prepare a new table derived from data obtained with the apparatus that will be used in all

CONTENT OF VOLATILE FATTY ACIDS IN FOUR FRACTIONS OF DISTILLATE

Designation	Distillate Fraction	Formic Acid	Acetic Acid (A)	Propionic Acid (P)	n-Butyric Acid (B)	i-Butyric Acid ($I-B$)
	ml.	%	%	%	%	%
T_1	50	11.3	19.3	33.9	48.9	62.2
T_2	200	33.8	46.5	53.3	47.4	37.0
T_3	200	20.9	19.6	10.3	3.4	0.8
T_4	200	12.9	8.3	2.0	0.3	0.0
$T_2/T_1 = C$		2.99	2.41	1.57	0.97	0.59

subsequent analyses, following exactly the same details of manipulation.

In all the following equations, A, P, B, and $I-B$ designate milliliters of 0.05 N acetic, propionic, butyric, and iso-butyric acids respectively:

Single-Acid System. If the acid is known, collect and titrate one 50-ml. fraction (T_1) of distillate and one 200-ml. fraction (T_2). If the identity of the acid is not known, compute the C from T_1 and T_2 respectively and identify by comparing with the table.

Two-Acid System. Steam distil the mixture as above, collect, and titrate 50- and 200-ml. fractions (T_1 and T_2). Then from the table

$$T_1 = 0.193A + 0.489B\ *$$
$$T_2 = 0.465A + 0.474B\ *$$

Solving for A and B:

$$A = 3.475T_1 + 3.593T_2$$
$$B = 3.417T_1 - 1.418T_2$$

Three-Acid System. Collect one 50-ml. and two 200-ml. portions. Assume only acetic, propionic, and butyric acids are present. Then from the table:

$$T_1 = 0.193A + 0.339P + 0.489B$$
$$T_2 = 0.465A + 0.533P + 0.474B$$
$$T_3 = 0.196A + 0.103P + 0.034B$$

* The numerical coefficients of A and B may be somewhat different in another distillation.

Solving for A, P, and B:

$$A = 4.116T_1 - 5.211T_2 + 13.410T_3$$
$$P = -10.342T_1 + 11.977T_2 - 18.232T_3$$
$$B = 7.589T_1 - 6.247T_2 + 7.347T_3$$

Four-Acid System. Determine formic acid in three portions of distillate combined (450 ml.) and compute the total formic acid present in the distilling flask at the start. Correct 50-ml. and 200-ml. portions for formic acid present and substitute the corrected titration in the equations for the other three acids.

$$T_1 = 0.193A + 0.339P + 0.489B + 0.622I - B$$
$$T_2 = 0.465A + 0.533P + 0.474B + 0.370I - B$$
$$T_3 = 0.196A + 0.103P + 0.034B + 0.008I - B$$
$$T_4 = 0.083A + 0.020P + 0.003B + 0.000I - B$$

Solving for A, P, B, and $I-B$,

$$A = -0.383T_1 + 0.830T_2 - 8.574T_3 + 28.537T_4$$
$$P = 2.702T_1 - 5.525T_2 + 45.463T_3 - 82.686T_4$$
$$B = -7.404T_1 + 13.871T_2 - 65.864T_3 + 95.042T_4$$
$$I - B = 6.075T_1 - 8.151T_2 + 29.664T_3 - 38.509T_4$$

Formic and Acetic Acids

Fuchs Mercuric Chloride Volumetric Method.[113] The basic reaction is

$$NaCHO_2 + 2HgCl_2 \rightarrow NaCl + Hg_2Cl_2 + HCl + CO_2$$

and the final step is the titration of the liberated hydrochloric acid.

PROCESS. *Preliminary Titration.* Place the weighed or measured portion, corresponding to 1 to 2 g. of the water-free acids, in a 100- or (if the amount of formic acid is large) 200-ml. volumetric flask. Titrate the diluted solution with *1.0 N sodium hydroxide solution,* using *phenolphthalein indicator,* boil to remove carbon dioxide, and complete the titration with a few drops of the *standard alkali.* Again boil to remove the trace of acetaldehyde which may be formed by the action of the acetic acid on acetylene and again adjust to the neutral point. Record the total volume of 1.0 N alkali required as N.

Mercuric Chloride Precipitation. To the neutralized solution add *solid sodium acetate* and *concentrated mercuric chloride solution* in liberal excess, then sufficient water to fill the flask nearly three-quarters. Heat until carbon dioxide is copiously formed and continue the heating nearly to the boiling point on the water bath or with a small flame for 15 minutes, avoiding loss from foaming. Cool to room temperature, fill to the mark, mix, filter on a dry paper, and pipet a 50-ml. aliquot into a porcelain casserole.

Final Titration. Taking advantage of the presence of the excess of mercuric chloride which serves as an indicator, titrate with *1.0 N sodium hydroxide* (0.5 N for a small formic acid content) the hydrochloric acid formed in the reaction until the color changes to bright yellow. Addition of a few additional milliliters of the *mercuric chloride solution* may be necessary to bring about a marked color change.

CALCULATION. Obtain the content of formic acid (F) and of acetic acid (A) in grams per 100 ml. of the solution before aliquoting by the following formulas:

$$F = n \times 0.04602$$

$$A = (N - n) \times 0.06003$$

in which N and n are the milliliters of N alkali required respectively for the preliminary titration and, calculated for 100 ml., final titration.

Formic Acid

Fincke Mercuric Chloride Gravimetric Method.[114] The original method was designed for vinegar; as here given, using somewhat different reagents, it is an Official method. Mercuric chloride is reduced to the mercurous salt.

APPARATUS. A *train* consisting of (1) a *steam-generating flask,* with a funnel tube extending to the bottom, (2) a *distilling flask,* with a spray trap in the outlet tube, (3) an *absorption flask,* with a perforated bulb at the bottom of the inlet tube to distribute bubbles, (4) an *upright condenser,* and (5) a *receiving flask.*

REAGENT. *Mercuric Reagent.* Dissolve 100 g. of $HgCl_2$ and 150 g. of NaCl in water, dilute to 1 liter, and filter.

PROCESS. *Distillation.* Place in the absorption flask 2 g. of *barium carbonate* suspended in 100 ml. of water. If much acetic acid is present, use an amount of the carbonate such that there will be 1 g. in excess. Into the distilling flask pipet 50 ml. of a fruit juice or other thin liquid, or weigh 50 g. of a sirup, jam, or jelly, and dilute with an equal volume of water. Add 1 g. of *tartaric acid* and connect with the train. Bring the contents of the distilling and absorption flasks to boiling and distil with steam until the distillate measures 1 liter, maintaining the volume in the distilling and absorption flasks

constant by heating, avoiding charring of the sample.

Precipitation of Mercurous Chloride. Filter the contents of the absorption flask while hot, wash the *barium carbonate* with a little hot water, and adjust the volume of the filtrate to about 150 ml. by dilution or evaporation. Add 10 ml. of *50% sodium acetate solution,* 2 ml. of *10% hydrochloric acid* and 25 ml. of the *mercuric reagent.* Mix well and immerse the container in boiling water for 2 hours. Collect the precipitate of mercurous chloride in a tared Gooch crucible, wash thoroughly with cold water and finally with a little *ethanol.* Dry in a boiling water oven for 30 minutes, cool in a desiccator, and weigh. If the weight exceeds 1.5 g., repeat the determination, using more of the *mercuric reagent.* Conduct a blank determination on the reagent and introduce the correction.

CALCULATION. Multiply the weight of mercurous chloride by the factor 0.0975, thus obtaining the weight of formic acid.

Hanak and Kürschner Permanganate Colorimetric Method.[115] The figures submitted indicate that the method is more accurate than the mercuric chloride and the gasometric methods.

APPARATUS. *Colorimeter.*

PROCESS. *Distillation.* Steam distil 10 ml. of the sample with 0.3 g. of *tartaric acid,* regulating the flow so that in 45 minutes the volume in the distilling flask has not increased more than 20 ml. To prevent overheating place the flask over a hole 4.5 cm. in diameter cut in a piece of asbestos board which rests on a piece of wire gauze. Add a small amount of water to the receiver at the start and take care that the delivery tube always dips beneath the liquid. After collecting 400 ml. of distillate, transfer to a 500-ml. volumetric flask, fill to the mark, and mix.

Titration. Titrate a 200-ml. aliquot with *standard alkali* for the determination of the total free volatile acids.

Saponification. Remove another aliquot of 100 or 200 ml. (depending on the formic acid content) to a saponification flask, add 0.5 to 1 ml. of *0.5 N potassium hydroxide solution* in addition to that required to neutralize the free volatile acids, as calculated from the titration, and saponify the formic esters by heating for 10 minutes. Transfer to a beaker, rinsing with water, add 0.2 g. of *sodium carbonate,* and evaporate to about 30 ml., avoiding loss from spattering by keeping the heat below boiling.

Color Formation with Permanganate. Add to the solution sufficient *0.2 N potassium permanganate solution* to leave 0.5 to 1.5 ml. in excess, 3 ml. usually being sufficient for 100 ml. of the original solution and 5 ml. for 200 ml. Dilute a measured volume with an equal volume of water if the content of formic acid is low and the color produced by the permanganate is too intense for accurate reading. After standing 45 to 60 minutes in a covered beaker, add 1 ml. of *10% zinc sulfate solution,* thus flocking the permanganate precipitate. Decant the supernatant liquid into a 50-ml. volumetric flask, rinse, fill to the mark, and filter through a porcelain Gooch crucible.

Color Comparison. Use for the comparison 25 ml. of the unknown and match against *0.2 N potassium permanganate solution.*

CALCULATION. Use: 1 ml. of 0.2 N $KMnO_4$ = 2.76 mg. of formic acid.

Grossfeld and Payfer Perforation Gravimetric Method.[116] The tedious procedure combines extraction (perforation), distillation with phosphoric acid, and precipitation of mercurous chloride by the reaction of formic acid with mercuric chloride.

APPARATUS. *Perforator.* The assembly employed (furnished by Schott, Jena) is for extraction of the aqueous solution with ether. The Erlenmeyer extraction flask contains ether and calcium oxide. The perforator proper (extractor) is placed beneath the

vertical condenser with an upper layer of ether and a lower layer of aqueous solution. The ether is delivered into the slender funnel tube and is broken up into minute bubbles by the sintered-glass plate at the recurved end.

REAGENTS. *Phosphotungstic Acid Reagent.* Dissolve 60 g. of sodium phosphate and 100 g. of sodium tungstate in water and dilute to 1 liter.

Mercuric Reagent. Dissolve 10 g. of $HgCl_2$, 4 g. of NaCl, and 10 g. of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) in water and dilute to 100 ml. Heat in a boiling water bath for 1 hour. Cool and filter through a pleated paper.[117]

PROCESS. *Clarification.* Weigh a portion of the sample, containing up to 100 mg. of formic acid, into a 250-ml. volumetric flask. A suitable amount for honey is 125 g., for fruit products a somewhat smaller amount. Dilute to 150 to 200 ml., add 20 ml. of *1 + 3 sulfuric acid* and 5 ml. of *15% potassium ferrocyanide solution*, shake, add 5 ml. of *30% zinc sulfate solution*, mix again, fill to the mark, and filter through a dry paper on which a pinch of *Kieselguhr* has been sprinkled.

Test 5 to 10 ml. of the filtrate by shaking with an equal volume of *ether*. If proteins are still present (this is unusual), as indicated by an emulsion which will appear between the two layers, transfer 200 ml. of the filtrate to another 250-ml. flask, add 25 ml. of *phosphotungstic acid reagent,* fill to the mark, and let stand overnight, then filter through a dry pleated paper.

Perforation. Transfer 200 ml. of the filtrate, whether after the first or the second precipitation, to the extraction chamber of the perforator and add *ether* up to the delivery tube to which previously 0.5 g. of *calcium oxide* and about 50 ml. of *ether* had been added. Heat for 5 to 6 hours on an electric plate at a rate such as the size of the apparatus and the bumping permit.

After the perforation is complete, remove the Erlenmeyer flask and cautiously distil off the ether on a water bath. Stir the residue with 10 to 15 ml. of water, add a pinch of *animal charcoal,* let stand 5 minutes, filter into the 250-ml. Erlenmeyer flask used for the distillation, and wash with water. Evaporate the filtrate together with *granular pumice,* at first on a hot plate and finally in a boiling water bath.

Distillation of Formic Acid with Naphtha. To the residue in the Erlenmeyer flask add 5 ml. of water, 1 ml. of *25% phosphoric acid,* and 150 ml. of *naphtha* (b.p. 80 to 90°). Distil into a graduated cylinder containing 15 ml. of water at such a rate that the drops cannot be counted. When 3.5 ml. of the aqueous phase have passed over and the contents of the flask have taken on a yellow color, interrupt the distillation, add 5 ml. of water to the flask, and continue the distillation until 5 ml. more of aqueous distillate are obtained. Repeat the addition of 5 ml. of water and collection of aqueous distillate until at least 19.5 ml. of total aqueous distillate are obtained. Let stand 10 minutes, remove the aqueous phase to a dish, add the rinsings of the condenser together with 1 g. of *calcium carbonate,* and evaporate to dryness.

Mercurous Chloride Precipitation. Moisten the residue with water, transfer with water to a filter tube (18 x 2.5 cm.), and wash with water until the filtrate measures 50 ml. Add 15 ml. of *mercuric reagent* and heat 1 hour in a boiling water bath. Collect the precipitate of mercurous chloride in a glass filter crucible (Schott and Gen. 10 G 3), dry at 105°, and weigh.

CALCULATION. Multiply the weight of the mercurous chloride by 97.5 to obtain the corresponding weight of formic acid.

EXAMPLES. Honey 2.9 to 5.8, cherry juice 218.5 to 242.5, strawberry juice 293.9, currant juice 6.6, and blood orange juice 2.8 mg. per 100 ml.

Acetic Acid

See Part II, D3.

Lactic Acid

See also Part II, C3, F1, G1, and H1.

Boisson Periodic Acid Volumetric Method.[118] Apparatus. An assembly of a *200-ml. flask* (*A*) provided with a ground joint *air-inlet tube* (*D*), reaching to the bottom of the flask, and a vertical *fractionating column* joined by a ground adapter to a long tube bent downward into an *Erlenmeyer flask* (*B*), acting as an absorber, which in turn is connected with a second *absorption flask* (*C*).

Reagents. *Sodium Periodate Reagent.* Prepare 10% $NaIO_4$ in 1.0 *N* H_2SO_4.

Mercuric Reagent. Dissolve 27 g. of $HgCl_2$ and 72 g. of KI in water and dilute to 250 ml.

Process. *Periodate Treatment.* Place in *A* 10 ml. of a solution of the sample containing 10 to 100 mg. of lactic acid.

(If dextrose is present, add *sulfuric acid* to about 0.2 *N* and *ammonium sulfate* to approximate saturation and extract with *ether* in a continuous extractor, then boil the extract for 2 hours on a water bath with 10 ml. of *0.2 N sodium hydroxide solution* to remove the lactic acid from the ether.)

Add to the solution 10 ml. of *sodium periodate reagent* and 2 ml. of *10 N sulfuric acid.* Prepare a mixture of 15 ml. of *sodium hydroxide solution* and 10 ml. of *mercuric reagent* and place 5 ml. of the mixture in the second absorption flask (*C*) and the remainder together with 5 ml. of *20% barium sulfate suspension* in the Erlenmeyer flask (*B*). Boil gently for 30 minutes while passing air through the air-inlet tube (*D*) at the rate of 10 to 20 liters per hour; this removes all the acetaldehyde as formed and permits its absorption.

Titration. Acidify the combined liquids from *B* and *C* with 25 ml. of *20% by volume sulfuric acid,* add a measured excess of *0.1 N iodine solution,* let stand with occasional shaking until the mercuric salt has dissolved, and titrate the excess of iodine with *standard 0.1 N sodium thiosulfate solution.*

Calculation. Use: 1 ml. of 0.1 *N* iodine solution = 4.5 mg. of lactic acid.

Oxalic Acid

The important role played by oxalic acid and oxalates in vegetable physiology warrants careful studies of methods and a greater accumulation of analytical data on a wide variety of vegetable foods than are recorded in the literature.

Kohl's book [119] is largely devoted to describing and picturing crystals of calcium oxalate in its highly characteristic forms. These crystals are conspicuous in microscopic preparations of fruits and vegetables, although the percentage amounts of oxalic acid, free and combined, even when calculated to the dry basis, is so small as to demand micro- or semi-micro methods for their determination.

The best-known methods are those of Bau and of Leulier *et al.*, which are adaptations of the gravimetric calcium oxalate method and the permanganate volumetric method respectively.

Bau Calcium Oxalate Gravimetric and Volumetric Methods. Bau (Bremen) reports results on cereals and beer,[120] also on tea, coffee, jams, vegetables, and other foods.[121] Tannin in tea must be removed.

Reagent. *Calcium Acetate Reagent.* (*a*) Dissolve 330 g. of $NaC_2H_3O_2 \cdot 3H_2O$ in warm water, cool, and dilute to 500 ml. (*b*) Dissolve 25 g. of $CaCl_2$ in and make up to 500 ml. with 50% acetic acid. Mix (*a*) and (*b*), let stand 48 hours at about 7°, and filter.

Process. *Extraction.* Prepare the solution for the determination of free or combined oxalic acid by maceration with water or dilute hydrochloric acid respectively in an atmosphere of carbon dioxide.

To remove the disturbing influence of tartaric acid, treat the solution containing not more than 0.2% of oxalic acid with ¼ mole of *boric acid* per mole of tartaric acid and one-fifth its volume of *calcium acetate solution*, then let stand 44 hours in the refrigerator.[122]

Calcium Oxalate Precipitation. Add to an aliquot of the extract one-fifth its volume of the *calcium acetate reagent;* let stand at about 7° in the refrigerator for 38 to 44 hours. Filter and measure the volume of the filtrate, then wash the precipitate until chlorine is removed and measure the volume of the wash liquid.

(*a*) *Gravimetric Procedure.* Ignite the paper and precipitate, then weigh.

(*b*) *Titration.* Dissolve the precipitate in *0.1 N hydrochloric acid* and titrate back with *0.1 N sodium hydroxide solution,* using *methyl orange indicator.* If considerable manganese is present, employ *0.1 N nitric acid.*

CALCULATION. Use: 1 ml. of 0.1 N acid = 4.40 mg. of C_2O_4. Correct for the solubility of the precipitate in the filtrate (1 liter = 3.34 mg. of C_2O_4) and wash liquid (1 liter = 4.54 mg. of C_2O_4).

NOTES. Preserve the solution of the sample with *toluene;* avoid light, enzymes, animal charcoal, high concentrations of SO_4^{--}, Mg^{++}, and Mn^{++++}, and P_2O_5, and Fe together, and more than 1% of citric and of tartaric acid. If the precipitate is slimy, make strongly acid with *hydrochloric acid* and salt out with *ammonium chloride.*

Saburov, Kalebin, Khakhina, and Danilova,[123] who found that the method gives more accurate results in the presence of tartaric, citric, and malic acids than the Butkevich method, state that pectin must be removed by precipitation.

Leulier, Velluz, and Griffon Calcium Oxalate-Permanganate Volumetric Method.[124] Leulier and Dorche[125] adapted the method to the determination of oxalic acid in urine and other liquids containing as little as 10 mg. per liter. The original method is as follows.

PROCESS. *Extraction.* Prepare the solution as in the Bau method, acidify (if not already acid), and extract with ether for 72 hours in a continuous extraction apparatus. Separate the ether layer and evaporate to dryness.

Calcium Oxalate Precipitation. Place the aqueous solution (3 to 5 ml.) containing the oxalic acid in a 12- to 15-cm. centrifuge tube, add 2 ml. of *saturated calcium hydroxide solution,* and let stand for 6 hours in ice water or for 12 hours at room temperature. Add to the solution 0.5 ml. of *0.5% ammonium phosphate solution,* stir cautiously for 5 minutes, and centrifuge for 2 to 3 minutes. Decant the supernatant liquid and wash twice with 1 ml. of cold water, centrifuging and decanting after each addition.

Titration. To the precipitate in the tube add 5 drops of *20% sulfuric acid,* 2 drops of *1% manganese sulfate solution,* and exactly 2 ml. of *0.6% potassium permanganate solution,* then stir gently, let stand 3 to 5 minutes, and add 3 drops of colorless *10% potassium iodide solution.* Titrate back with *0.01 N sodium thiosulfate solution* over a white surface, comparing with pure water (A). Titrate in like manner 2 ml. of the 0.3% permanganate solution (B).

CALCULATION. Obtain the milligrams of oxalic acid (O) by the formula

$$O = 0.45(B - A)$$

EXAMPLES. In solutions containing 0.225 to 0.562 mg. of oxalic acid, the errors ranged from −3.1 to +2.7%.

PYRUVIC ACID

Simon Nitroprusside Colorimetric Method.[126] Finding that the intense color obtained in the Legal reaction is not specific, Simon based a method on the blue color formed with nitroferricyanide in conjunction with ammonia and, for special delicacy, a lit-

tle acetic acid. It is claimed that acetaldehyde does not interfere, also that the same color, obtained with acetophenone is characterized by being formed with the reagents in the presence of potassium hydroxide. Acetone gives a rose-violet color and acetoacetic ester an orange color, but both colors are much less intense than that formed with pyruvic acid.

Apparatus. *Duboscq Colorimeter.*

Process. *A. Preliminary Test.* To 1 ml. of the extract of the sample add 0.5 ml. of *40% acetic acid*, 3 ml. of freshly prepared *1% sodium nitroferricyanide solution*, and 1.5 ml. of *1 + 1 ammonium hydroxide.* The appearance of a blue color in 2 minutes is indicative of about 1% of pyruvic acid and in 10 minutes of about 0.2%.

If no color appears, repeat the test, using 1 ml. of the *sodium nitroferricyanide solution.*

B. Quantitative Determination. If the blue color appeared within 5 minutes, use in the actual analysis 4 ml. of *nitroferricyanide solution* and compare the color formed with that of a *1% solution of pyruvic acid*, after allowing to stand 45 minutes. If, however, the color appeared after 5 minutes, use 2 ml. of *nitroferricyanide solution* and compare with the color formed with 0.3 to 0.4% of pyruvic acid after 1.5 hours.

Color Comparison. The limits for the Duboscq colorimeter are 0.2 to 1%. If the concentration is higher, dilute accordingly.

Note. Carpeniseanu [127] increases the sensitivity of the method about tenfold by using more dilute solutions and a greater number of standards.

De Jong Phenylhydrazine Volumetric Method Modified by Simon and Piaux.[128] Reagent. *Phenylhydrazine Reagent.* Dissolve 26 g. of phenylhydrazine in 50 ml. of hydrochloric acid and make up to 1 liter with water.

Process. Estimate the amount of pyruvic acid in the solution by the preliminary Simon Nitroprusside Test (see above), then add an excess of *phenylhydrazine reagent.* Filter after 3 to 4 hours, wash with water until no longer acid to *helianthine*, and measure accurately the volume of filtrate plus washings (V). Suspend the precipitate in a little water, add a few drops of *phenolphthalein*, and titrate with *standard sodium hydroxide solution.*

Calculation. Obtain the milligrams of pyruvic acid (W) in the solution by the following formula:

in which v is the volume of the standard alkali solution required, t is the gram moles per liter, and V is the volume as noted above.

Lieben Lactic Acid-Acetaldehyde Volumetric Method.[129] In the course of studies on the decomposition of pyruvic acid by yeast, Lieben developed a procedure for reducing the acid to lactic acid and oxidizing the latter by the Fürth and Charnass Method (which see).

Krishna and Sreenivasaya Modification.[130] Apparatus. A simplified *Friedmann Aeration Apparatus* for acetaldehyde (Fig. 57), developed at the Indian Institute of Science, Bangalore.

Process. *Reduction to Lactic Acid.* Treat 1 to 5 ml. of the solution of the unknown, containing 0.25 to 15 mg. of pyruvic acid, with 50 ml. of *17.5% sulfuric acid*, 0.5 to 1 g. of *zinc dust*, and 1 ml. of *10% cupric sulfate solution.*

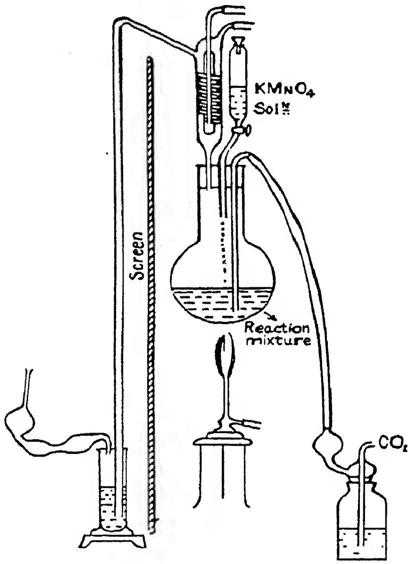
Oxidation to Acetaldehyde. After 1 hour, filter into the reaction flask and neutralize slowly to *dimethylaminoazobenzene* with *60% sodium hydroxide solution* added dropwise, then add 10 ml. of *0.1 N manganese sulfate solution in 10 N sulfuric acid* and oxidize with *0.005 or 0.01 N potassium permanganate solution.*

Aeration and Titration of the Bound Acetaldehyde. See Clausen Acetaldehyde Method for determination of lactic acid.

Kendall and Friedemann Zinc-Lactic Acid Method.[131] The pyruvic acid is reduced to lactic acid which is then determined by the method devised by the same authors.[132]

APPARATUS. *Lactic Acid Assembly.*

PROCESS. *Reduction.* Heat 75 ml. of the solution, containing not more than 60 or less



Courtesy of Biochem. J. 1928, **22**, 1171

FIG. 57. Krishna and Sreenivasaya Acetaldehyde Aeration Apparatus.

than 10 mg. of pyruvic acid, in a 250-ml. volumetric flask with 0.5 g. of *zinc dust*, 2.5 g. of *sodium bisulfate*, and 1 drop of *20% cupric sulfate solution* for 2 hours in a boiling water bath (2.5 ml. of hydrochloric acid may be substituted for the sodium bisulfate, but the result is lower). Remove from the bath, wash the neck, and cool.

Lactic Acid Determination. (*A*) If no interfering substances are present, nearly neutralize with *sodium hydroxide solution*, make up to the 250-ml. mark, and aliquot into a Kjeldahl flask for the lactic acid determination.

(*B*) If sugars, amino acids, etc., are present, cool the flask, nearly neutralize with *sodium hydroxide solution*, add 10 ml. each of *20% cupric sulfate solution* and *calcium hydroxide suspension*, make up to the 250-ml. mark, shake, and filter. Pipet an aliquot into a Kjeldahl flask and proceed with the lactic acid determination.

Straub Salicylaldehyde Colorimetric Method.[133] In connection with a series of respiration investigations, primarily directed to the role of fumaric acid, Straub determined pyruvic acid as follows.

APPARATUS. *Pulfrich Photometer.*

PROCESS. *Removal of Proteins.* Treat 4 ml. of muscle suspension with 0.5 ml. of *10% sulfuric acid* and 5 ml. of *10% sodium tungstate solution*, and filter.

Color Formation and Reading. To 1 ml. of the filtrate, containing not over 0.5 ml. of pyruvic acid, add 1 ml. of *10 + 6 potassium hydroxide solution* and 0.5 ml. of *2% salicylaldehyde solution in ethanol*, heat to 37°, and shake thoroughly for 10 minutes. Cool, centrifuge, and read the extinction in the Pulfrich photometer. Correct for the color formed in a blank determination.

CALCULATION. Since the color does not follow Beer's law, comparison with a calculation curve is essential.

NOTE. The interference of oxalacetic acid prior to the adding of potassium hydroxide is minimized by rapid manipulation, but the absorption of pyruvic acid by the tungstate precipitate is a considerable source of error. Even after correction, an average error of 10% is admitted. Delrue [134] avoids the loss of pyruvic acid in the tungstic precipitate by adding sufficient water to hold the precipitate in solution.

MALIC ACID

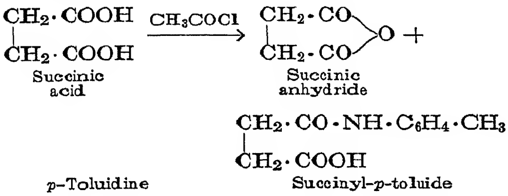
See Part II, D2.

Tartaric Acid

See Part II, D2, F1, and K2.

Succinic Acid

Pucher and Vickery Toluidine Gravimetric Method.[135] The method is an application of the following reactions discovered by Auwers:[136]



Reagent. *Succinyl-p-Toluide Wash Liquid.* Treat 0.5 g. of the recrystallized succinic anhydride with 0.6 g. of *p*-toluidine in 15 ml. of chloroform, warm to the boiling point for 30 minutes, then cool to 5°. Collect the crystals on a filter and wash with cold chloroform. Heat 50 mg. of the crystals (m.p. 178°) with 500 ml. of toluene for a short time, chill in the refrigerator overnight, filter, and store in the refrigerator.

Process. *Extraction.* Weigh 1 g. of the sample obtained by grinding the fresh material, dried at 80°, and extract with ether as described by Pucher, Wakeman, and Vickery.[137] Treat the ether extract with 25 ml. of water and evaporate the ether. Dilute the solution with 25 ml. of water, add 2 ml. of *5 N sodium hydroxide solution*, and agitate until all the residue has dissolved. Acidify with 3.5 ml. of *18 N sulfuric acid*, then add 5 ml. of cold *saturated barium hydroxide solution* and 0.5 g. of *asbestos.* Boil gently for a few minutes, cool, filter with suction through asbestos into a 150-ml. beaker with a mark showing 75 ml., and wash the flask and asbestos repeatedly with small amounts of water to the mark. Heat the filtrate for 10 minutes in a boiling water bath, add 30 to 35 ml. of *1.5 N potassium permanganate solution,* and continue the boiling for 30 minutes, testing by spotting on filter paper for an excess of permanganate and adding more if necessary. Treat the hot solution with freshly prepared *20% anhydrous sodium sulfite solution* until colorless, then add 0.5 ml. in excess.

Evaporate to about 25 ml. on the steam bath, filter into the tube of a continuous liquid extraction apparatus, and extract with *ethanol-free ether* overnight in a Pucher and Vickery[138] or Quick[139] extractor. Treat the ether extract with 5 ml. of water and evaporate most of the ether on the water bath, then boil the aqueous residue for a few minutes, cool, and wash into an Erlenmeyer flask with a standard taper joint, and evaporate to dryness on the steam bath.

To complete further the oxidation of all matter other than succinic acid, treat the residue with 0.2 ml. of *hydrochloric acid* and 0.1 ml. of *nitric acid* and again evaporate to dryness on the steam bath. Cool and keep overnight in a vacuum desiccator containing *solid sodium hydroxide* to remove all volatile acids.

Dehydration. Add to the dry residue of succinic acid 0.5 ml. of *redistilled acetyl chloride* (b.p. 50 to 54°) and reflux for 1 hour, shaking occasionally and protecting from moisture, in an oil bath at 55 to 60°. Cool for 3 to 4 minutes, then keep the flask overnight in a desiccator containing *sulfuric acid* and *solid sodium hydroxide* without exhausting.

Succinyl-p-Toluide Formation. Add to the flask 5 ml. of boiling *toluene*, cover with a glass bulb, maintain the boiling for 5 minutes, add 50 mg. of *tricalcium phosphate* as filter aid, and filter with gentle suction into a 10-ml. beaker through a thin layer of asbestos in a dry Gooch crucible. Wash the flask twice with 1 ml. and the crucible once with 0.5 ml. of boiling *toluene*. Treat the clear filtrate with 0.5 ml. of *6% solution of p-*

toluidine in toluene with stirring, remove the rod, rinsing with *toluene*, and let stand for 5 minutes, then warm in an oil bath to 60 to 65° for 10 minutes. Cool to room temperature for 10 minutes, then in water at 4 to 5°, and store in the refrigerator for 1 hour. After about 15 minutes insert a rod and stir occasionally.

Filtration. Collect the precipitate on a 1.5-ml. dry tared Gooch crucible with a thin asbestos mat, using a portion of the chilled filtrate to aid in the transfer. Wash twice with 1 ml. of *succinyl-p-toluide wash liquid* chilled in ice water. Dry the precipitate at 105° for 30 minutes and weigh.

CALCULATION. Add 0.34 to the weight of succinic-*p*-toluide and multiply the sum by 0.059, thus obtaining the percentage of succinic acid.

BUTYRIC, CAPROIC, CAPRYLIC, CAPRIC, AND LAURIC ACIDS

See Part II, B2(*b*), Reichert-Meissl Number.

CITRIC ACID

See Part II, D2, F1, and G1.

BENZOIC AND SALICYLIC ACIDS

See Part I, C13.

HYDROCYANIC ACID

See also Part II, C1 and J3*a*.

Although containing over 50% of nitrogen, hydrocyanic acid is grouped with the nitrogen-free acids for convenience. The amount present in food is so minute as to influence scarcely at all the nitrogen content.

By the hydrolysis of glucosides present in certain varieties of beans, particularly those grown in India, hydrocyanic acid is evolved. The residues from hydrocyanic acid sprays may contain cyanides.

Guignard Alkaline Picrate Test.[140] By the action of a cyanide on sodium picrate an orange-red coloration is produced. As developed by Morrow,[141] used by Greene [142] as a field test in a study of cyanogenetic glycosides in Arizona plants, and adopted as an official A.O.A.C. method, the technique is as follows.

REAGENT. *Picrate Test Paper.* Saturate filter paper with a 1% aqueous solution of picric acid, allow to dry, then dip in 10% $NaCO_3$ solution, and again dry. Keep in a well-stoppered bottle.

PROCESS. Place a suitable amount of the finely chopped sample in a test tube, add a few drops of *chloroform*, and insert a strip of the *picrate test paper* without allowing it to come in contact with the material. Stopper the tube tightly and let stand.

The presence of hydrocyanic acid is indicated by the formation of an orange coloration changing to brick red on standing.

Fox Iodide-Silver Test.[143] Greene [144] found this test more sensitive and specific than the Guignard alkaline picrate test.

PROCESS. Allow the chopped sample to autolyze with 4 or 5 parts by weight of water, then aspirate the gas into a drop of *5% potassium iodide solution* in a test tube. Add 1 drop of *0.001 M silver nitrate solution*, followed by 1 ml. of *5% sodium hydroxide solution*.

In the presence of hydrocyanic acid, the faint bluish cloud of silver iodide disappears and the solution becomes crystal clear.

Smith Alkaline Picrate Colorimetric Method.[145] The method (University of Chicago and University of Michigan) depends on the Guignard reaction (see above). The color appears when the solution contains as little as 0.011% of potassium cyanide. The following amounts of interfering substances must be present to give the colorations: ammonium sulfide 0.4, dextrose 0.4, acetaldehyde 15, acetone 20, and furfural 10 to 15%. Formaldehyde (40%) and acetoacetic ester

give colors too pale for comparison, and ethanol, methanol, ethyl acetate, and acetic acid no color.

APPARATUS. *Colorimeter.*

PROCESS. *Color Formation.* Pipet into a test tube with a 25-ml. graduation mark 3 ml. of *saturated picric acid solution,* 1 ml. of *5% sodium carbonate solution* (insuring an alkaline reaction), and 1 ml. of the solution of the unknown. Heat in a boiling water bath for 5 minutes, cool in running water, and dilute to the mark.

As a standard treat 1 ml. of *0.002 N cyanide solution* in like manner.

Color Comparison. Make the comparison of the unknown with the known solution set at 20 mm. in the colorimeter.

Möller and Stefansson Photometric Modification.[146] These authors publish from Copenhagen University.

APPARATUS. *Pulfrich Photometer.*

REAGENTS. *Standard Color Solution.* Into 4 test tubes of uniform diameter with marks at 25 ml., place aliquots of a stock solution corresponding to 25, 80, 120, and 600 γ of hydrocyanic acid and treat as in an actual analysis.

PROCESS. *Color Formation.* Pipet into a test tube with a mark at 25 ml. and in the order stated 10 ml. of *1% picric acid solution,* 1 ml. of *1.0 N sodium carbonate solution,* and 5 ml. of the unknown cyanide solution. Heat in a boiling water bath for 12 minutes, cool under the tap, and fill to the mark.

Carry along with the unknown a blank in which the cyanide solution is replaced by water. If the cyanide is separated from the sample by distillation, add 2 ml. of *2.0 N sodium carbonate solution*, instead of 1 ml. of 1.0 N, to 20 ml. of the solution.

In preparing the solution for photometric examination, the content of carbonate is important. If, for example, the distillate measures 20 ml., 5 ml. of that distillate will contain 1 ml. of 1.0 N carbonate.

Color Extinction. Select for the photometric examination a cell in which the absorption (D) will be between 5 and 60% and the extinction between 1.3 and 0.2. Use tube lengths for concentration as follows: 30 mm. for below 80 γ, 20 mm. for 25 to 100 γ, and 5 mm. for 80 to 600 γ. Place in the compensation cell (cuvet) the blank solution and employ filter S 53 (530 $m\mu$).

CALCULATION. Obtain the concentration (c) in terms of γ of hydrocyanic acid in 25 ml. of the color solution by the following formula:

in which E is the observed extinction and c/E is the ratio of concentration to extinction as calculated by interpolation in the table herewith. As in the original method, Beer's law holds only for concentrations 40 to 80 γ. In the original article a table is given also for a 20.06-mm. cell. It should be noted that on page 57 of Möller and Stefansson's article a/E is evidently a misprint for c/E; furthermore their use of "c" and "*E gemessen*" is confusing.

Schulek–Lang Bromination Iodometric Method.[147] PROCESS. *Bromination.* Pipet an aliquot of the unknown solution into a glass-stoppered 100-ml. flask with a neck 8 cm. long and quickly add 1 or 2 ml. respectively of *sirupy phosphoric acid* for 10 to 20 or 20 to 50 ml. of solution, then close the flask without delay. With one hand remove the stopper and with the other add *saturated bromine water* with gentle shaking until a yellow color persists, followed by *freshly pulverized ferrous sulfate* until the excess of bromine is removed, taking care to bring into solution any of the salt adhering to the neck. Rinse the stopper and neck with a few milliliters of water and mix with the least possible distribution of the solution over the sides of the flask.

Titration. Add 2 or 5 ml. of *10% potassium iodide solution* respectively per 1 or 2

STANDARDS FOR PULFRICH FILTER S 53 (MÖLLER AND STEFANSSON)

30.05-mm. Cell						5.03-mm. Cell		
E	*c/E*		*E*	*c/E*		*E*	*c/E*	
0.08	130 *		0.24	81.1		0.2	398 †	
		16			0.8			14
0.09	114		0.26	80.3		0.3	412	
		9.2			0.8			15
0.10	104.8		0.28	79.5		0.4	427	
		4.8			0.7			14
0.11	100.0		0.30	78.8		0.5	441	
		4.2			1.4			15
0.12	95.8		0.35	77.4		0.6	456	
		2.8			1.2			15
0.13	93.0		0.40	76.2		0.7	471	
		2.0			1.7			14
0.14	91.0		0.50	74.5 ‡		0.8	485	
		1.9			1.0			15
0.15	89.1		0.60	73.5		0.9	500	
		1.5			0.7			15
0.16	87.6		0.70	72.8		1.0	515	
		1.3			0.4			14
0.17	86.3		0.80	72.4		1.1	529	
		1.2			0.1			15
0.18	85.1		0.90	72.3		1.2	544 §	
		1.1			0.1			15
0.19	84.0		1.00	72.2		1.3	559	
		0.9			0			14
0.20	83.1		1.10	72.2		1.4	573	
		1.1			0			15
0.22	82.0		1.20	72.2 ‖		1.5	588	
		0.9						15
0.24	81.1					1.6	603	
								14
						1.7	617 ¶	

* $c = 0.08 \times 130 = 10.4\ \gamma$ HCN.
† $c = 0.2 \times 398 = 79.6\ \gamma$ HCN.
‡ $c = 0.5 \times 74.5 = 37.2\ \gamma$ HCN.
§ $c = 652\ \gamma$ HCN.
‖ $c = 1.2 = \times 72.2 = 86.6\ \gamma$ HCN.
¶ $c = 1050\ \gamma$ HCN.

ml. of *concentrated phosphoric acid* previously added, a little *starch solution,* and titrate with *standard 0.1 N sodium thiosulfate solution.*

If the amount of hydrocyanic acid exceeds 4 to 5 mg., add double the amount of *potassium iodide,* but do not increase the amount of phosphoric acid.

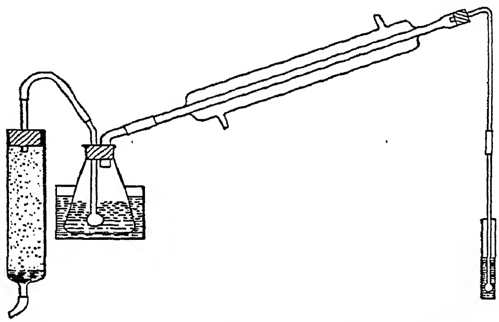
In the analysis of organs and similar tissues, distil and collect the distillate in 2 ml. of *2.0 N sodium hydroxide solution.* The neutralization of 0.2 ml. of phosphoric acid by this addition need not be taken into account, since considerable variation in the strength of the latter is allowable.

CALCULATION. Use: 1 ml. of 0.1 *N* $Na_2S_2O_3$ = 1.351 mg. of HCN.

Labatti Iodide-Silver Nitrate Volumetric Method.[148] The steam distillation procedure has been found less satisfactory than the following modification developed at the U. S. Food and Drug Control Laboratory.

I. Winkler Modification.[149] APPARATUS. *Aeration Assembly* (Fig. 58). The parts are (1) a soda lime tower connected at the bottom with a compressed air supply, (2) a bath containing a 300-ml. Erlenmeyer flask carrying a double-bored stopper through one hole of which passes a bulbous spray tube and through the other a tube connected with a diagonal reflux condenser, and (3) a 15-mm. flat-bottom absorption tube containing an alkaline solution into which a second bulbous spray tube extends nearly to the bottom.

PROCESS. *Aeration.* Place 11 ml. of water and 2 ml. of *10% sodium hydroxide solution* in the absorption tube. (For very small amounts, use only 1 ml. of *alkali* and 8 ml. of water.) Adjust the compressed air outlet so as to deliver 800 to 1000 ml. per minute, as tested by collecting the air over water. Weigh 25 g. of the ground or chopped sample into the Erlenmeyer flask, add 180 ml. *1 + 50 sulfuric acid,* close with a solid stopper, and shake well. Rinse the stopper with a little water and connect with the double-bored stopper. Immerse in the bath, as shown, to a depth of 2.5 to 4 cm. in *35% glycerol* and heat the bath rapidly to about 104°, then lower the flame to maintain a temperature of 105 to 110°. After the temperature of the bath reaches 100°, continue the flow of air for 15 minutes, then remove the flame and disconnect the air from the distillation flask. Disconnect also the spray tube in the absorption solution, but allow it to remain as a stirrer.



Courtesy of J. Assoc. Official Agr. Chem. 1939, **22,** *350*

FIG. 58. Winkler Hydrocyanic Acid Apparatus.

Titration. Add to the absorption tube 2 ml. of *1 + 2 N ammonium hydroxide* and 1 ml. of *5% potassium iodide solution* and titrate with *0.02 N silver nitrate solution* to the appearance of a turbidity as seen against a black background.

CALCULATION. Use: 1 ml. of 0.02 *N* $AgNO_3$ = 1.08 mg. of HCN.

EXAMPLES. A recovery of from 97.3 to 105.6% was obtained by Winkler, from a solution containing 1 to 40 mg. of potassium cyanide.

II. Ol'shevskaya Modification.[150] PROCESS. *Distillation.* Mix 100 g. of the finely powdered sample with 500 ml. of water, add 50 ml. of *10% tartaric acid solution,* and distil into 15 ml. of *0.5 N potassium hydroxide solution,* diluted with 15 to 20 ml. of water, until 300 to 500 ml. have passed over.

Titration (Libich Method). Add to the distillate a few drops of *10% potassium iodide solution* and titrate with *standard 0.1 N silver nitrate solution* to a permanent turbidity.

Higher Fatty Acids

See Part II, B2.

Tannins

Products containing considerable amounts of bitter substances of the tannin group are unfit for food. Methods for the determination of the tannins in tea, coffee, and cocoa are given in Part II, I, 1, 2, and 3, and in spices in Part II, J1.

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7. FIBER

See C2*e* above.

a. CELLULOSE

Several authors[1] remove lignin and cutin from the crude fiber with *oxidizing mixtures*, but the treatment does not dissolve the pentosans. König[2] removes pentosans, but not lignin and allied substances, by autoclaving 3 g. of the sample at 3 atmospheres (137°) for 1 hour with 200 ml. of *glycerol* (sp.gr. 1.23) containing 20 g. of sulfuric acid.

b. LIGNIN

Willstätter and Zechmeister Fuming Hydrochloric Acid Gravimetric Method;[3] Phillips and Goss Modification.[4]

APPARATUS. A train consisting of (1) a *1500-ml. salt-mouth bottle* containing 500 ml. of H_2SO_4, closed by a double-bored rubber stopper carrying (*a*) a 250-ml. globular separatory funnel with a long stem, narrowed and recurved at the end, extending to the bottom of the bottle and (*b*) an outlet tube, (2) an *all-glass Dreschel high-form gas-washing bottle*, (3) a *wooden cooling box* containing three Pyrex test tubes (300 mm. × 38 mm.), each with a double-bored rubber stopper carrying a long inlet tube and a short outlet tube provided with stopcocks and joined to the outlet tube of 2 and the inlet tube of 4 by three-pronged manifolds, and (4) a *500-ml. salt-mouth absorption bottle* with an inlet tube extending halfway to the bottom just above the water level.

REAGENT. *Fuming Hydrochloric Acid.* Treat 500 g. of NaCl in a liter Pyrex distilling flask with 250 ml. of water and 450 ml. of H_2SO_4, connect the side tube with an H_2SO_4 wash bottle and the latter with a flask containing 3 liters of HCl surrounded by crushed ice. Heat gently the distilling flask and pass the dry HCl gas into the solution of the acid until it reaches sp.gr. 1.212 to 1.223 at 15°. Store at 0° or below.

PROCESS. *Charge.* Place 1 g. of the sample in each of the 3 Pyrex test tubes, add 20 ml. of the *reagent* to each, and mix, then 30 ml. more and 3 drops of *capryl alcohol.* Pack the test tubes with crushed ice and lubricate the tubes entering the stoppers with glycerol.

Gas Treatment. Deliver *hydrochloric acid* from the separatory funnel into the sulfuric acid, lead the gas for 2 hours through sulfuric acid in the gas-washing bottle into the Pyrex test tubes, increasing the flow from slow to rapid during the last 15 minutes. Raise the inlet tubes, remove the test tubes, and store at 8 to 10° for 24 hours.

Cohobation and Filtration. Transfer the contents of the test tubes to 1-liter flasks, dilute to 500 ml., and reflux for 1 hour. Collect the crude lignin in tared Gooch crucibles, wash with hot water, dry at 105°, cool, and weigh.

Corrections. Determine the ash and the protein (N × 6.25) in two of the three crucibles and (if desired) the methoxyl in the third.[5] Correct the weight of crude lignin accordingly.

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8. ASH

(Mineral Constituents)

a. Principal Inorganic Elements

See Part I, 2f, for methods of determination of total ash, water-soluble ash, acid-insoluble ash (sand), and alkalinity of the ash.

Content of Ash Constituents in Typical Foods. A concise statement of the actual or relative amount of the several ash constituents is difficult, owing to the variation in different foods and different lots of the same food. In general, *potassium* is the chief basic mineral constituent of vegetables and meat, often reaching (calculated as K_2O) 30% of the ash and 0.5% or, in some oil seeds, 1.0% of the food; in milk, however, the content of calcium may equal that of *potassium*. *Magnesium* is more abundant than calcium in cereals and most oil seeds, but seldom approaches potassium in amount. Phosphorus is the chief acid-forming inorganic element, although largely in organic combination. *Silicon* in oats and some other chaffy cereals may be present in double the amount of the *phosphorus*, but this is due to the hulls which are not edible for humans. *Chlorine*, like *sodium*, is characteristic of sea foods and is an important constituent of milk. *Sulfur*, as found in the ash, is derived largely from sulfur organically combined in the food and should not be regarded as an acid constituent. The *carbon dioxide* (CO_2) of the ash is derived from organic acids or other organic constituents of the foods and, like sand, is not a constituent of the pure ash.

Careful analyses which are of value today were made about the middle of the last century by Way and Ogston in England and Wolff in Germany. They, as do many chemists today, express their results as percentages of the oxides or anhydrides, thus permitting a summation approximating 100, with due correction for the chlorine. The oxide system has the added advantage of employing terms that more nearly correspond to the forms actually present in the solutions and precipitates.

A study of the selected analyses given in subsequent chapters shows how little of the vital elements *calcium* and *phosphorus* is present in white flour and polished rice, and their practical absence in refined sugar. The results on *iron* in the general analyses are not of sufficient accuracy for forming conclusions, hence dependence must be placed chiefly on figures given under Minor Mineral Constituents below. It seems unfortunate that *sodium* occurs in land plants in such small amount, relative to that of *potassium*, when the demand of the body appears so urgent; lower animals, however, thrive on the amounts in natural foods.

Scheme for Complete Ash Analysis

In the strict sense, a complete analysis includes figures for the minor as well as the commoner constituents. Doubtless after a further study of methods, the scheme may be so amended as to be more comprehensive, but for the present for various reasons it seems desirable to keep major or gross constituents apart from minor or trace constituents.

Process. The scheme consists of a combination of well-known methods and a special method for the separation and determination of iron oxide, alumina, and lime in the presence of an excess of phosphoric acid as adapted from one developed by the late Professor S. L. Penfield of Yale University for mineral analysis.

Preparation of Ash. Regulate the amount of material burned according to the percentage of ash judged to be present. Dry fresh vegetables, fruits, and other succulent foods in a suitable arrangement, such as a tray over a floor register, determine the loss in weight, grind or chop, mix, and weigh out a suitable amount (50 to 200 g.) of the resultant sample into a platinum or quartz dish for incineration and analysis. If the approximate ash content is not known, make a preliminary determination. Carry out the ashing at a heat below redness, weigh the ash, and calculate the percentages in the air-dry and the fresh material.

Ordinarily about 2.5 g. of ash are sufficient for a complete analysis, depending on another ashing for a duplicate analysis or certain check determinations. A skillful manipulator with a sensitive balance will complete a dependable analysis on as little as 1 or even 0.5 g. of ash, but this requires some ingenuity in so adapting the scheme as to make more determinations on the same weighed portion. By dissolving and reanalyzing each precipitate after weighing and by the footing of the analysis, he will establish the accuracy of his figures. A footing within 0.50% of 100.00 (on the oxide basis) is possible, although one within 0.75% is usually rated as sufficiently accurate, provided this error does not fall on a constituent present in small amount.

Particularly troublesome on combustion are starchy cereal products which do not contain an excess of basic mineral constituents and consequently on burning yield an ash that is free from carbon dioxide. In preparing the material for ash analysis, it is better to have an ash containing considerable carbon, which is determined as one constituent of the ash, than to resort to treatment with nitric acid during incineration, since this treatment is injurious to platinum dishes and fatal to chlorine and sulfur, if indeed they have not already disappeared entirely.

Whatever the substance or the method of obtaining the true ash, there is no assurance that the full amount of chlorine and sulfur passes into the ash, thus necessitating special treatment, such as burning with a carbonate as noted below, if these are to be determined with accuracy.

The errors of burning may not all be minus; if gas flames are depended on for heat, the ash is likely to be contaminated with sulfur fumes from the products of combustion. Substitution of alcohol for illuminating gas as fuel or incineration in an electric muffle furnace obviates this error.

Although some discretion must be exercised in conducting the ashing, three don'ts apply in all cases: Don't rush the process, don't exceed a dull red heat, and don't stir while the burning is slowly moving in a layer from just beneath the surface to the bottom of the dish; only when this progressive burning is complete is it advisable to stir in the surface layer of carbon. A small amount of carbon in the ash does not appreciably affect the solubility of the inorganic matter.

Portion of Ash for Analysis. If a single determination of each constituent is deemed sufficient, employ a 1-g. portion of ash: (A) for *water*, *carbon dioxide*, *charcoal*, *sand*, *silica*, *iron oxide*, *alumina*, *magnesia*, and *phosphoric acid;* and a second 1-g. portion (B) for *chlorine*, *sulfuric acid*, *potash*, and *soda*. As little as 0.5-g. portions are employed when the supply of ash is limited, in which case special care must be taken to secure accuracy. If the quantity of ash permits, determine chlorine and total phosphoric acid, each on separate 0.5-g. portions.

The steps of the procedure which follows are described under the heads of the constituents determined.

WATER

Dry 1 g. of the ash (Portion A), contained in a platinum crucible, over a flame at a heat well below redness. An alternate course is to raise the heat to dull redness so as to burn the carbon, thus determining the carbon and water together, both being extraneous con-

stituents of the pure ash. The former course is usually the preferable one, since it avoids fusion of the phosphates and the consequent difficulty of solution.

Carbon Dioxide

Use the portion dried for the determination of the water. Determine the total carbon dioxide as directed for baking powder (Part II, K2). If it is desired to determine chlorine as the next step, liberate the carbon dioxide with *nitric acid* free from oxides of nitrogen, otherwise use *hydrochloric acid* as directed.

Reserve the acid solution for the following determinations.

Carbon and Sand

Transfer the acid solution and the insoluble matter remaining after the determination of carbon dioxide to a beaker, adding more *hydrochloric acid* if necessary to make the acid content about 10%, and boil for a few minutes. Collect the carbon and sand on a Gooch crucible and wash with hot water, reserving the filtrate for subsequent determinations. Dry the Gooch crucible and contents at 110° to constant weight. Ignite until the carbon is entirely burned and again weigh, thus obtaining the sand.

Calculate the carbon by difference.

Silicon

Transfer the filtrate from the carbon and sand determination to a platinum dish, evaporate to full dryness on a steam bath, then cautiously increase the heat, and finally move a flame rapidly under the dish, avoiding redness. Add to the residue sufficient *hydrochloric acid* to moisten the salts thoroughly, allow to stand for a few minutes, then stir with several portions of hot water, decanting after each portion on an ashless filter. Transfer the silica to the filter when it is obvious that nothing else remains undissolved. Wash with hot water, ignite, and weigh in a platinum crucible.

Calculate as silicon (Si) or silica (SiO_2).

Iron and Aluminum

Precipitation as Phosphates. To the filtrate from the silica determination, contained in a beaker, add with stirring *ammonium hydroxide* until a precipitate forms that does not dissolve, avoiding a further excess, then *hydrochloric acid* sufficient just to clear the solution. Heat in a water bath at 50°, add *ammonium acetate solution* until a precipitate forms, then add a slight excess, and 4 ml. of *glacial acetic acid.* Continue the heating at 50° until the rather scanty precipitate of mixed iron and aluminum phosphates settles. Filter on an ashless paper, wash with hot water, ignite in a platinum crucible at a moderate heat, and weigh.

Iron Titration. Fuse the weighed mixed phosphates with at least 10 parts of *sodium carbonate,* dissolve in *dilute sulfuric acid,* reduce with *hydrogen sulfide,* titrate with *standard potassium permanganate solution* for the determination of the iron, and determine phosphoric acid in the same solution by the Sonnenschein molybdate-magnesium pyrophosphate method, below.

Aluminum Calculation. Deduct from the weight of the mixed phosphates the sum of the weights of the ferric oxide (Fe_2O_3) and the phosphoric acid (P_2O_5), thus obtaining the weight of the alumina (Al_2O_3); $Al_2O_3 \times 0.5291 = Al$.

Iron Calculation. When, as is almost invariably true, the percentage of alumina calculated to the ash is less than 0.1%, it is more accurate to calculate the weight of iron oxide by multiplying the weight of the mixed phosphates by 0.53 (the factor for ferric phosphate), ignoring the alumina which may be determined by a colorimetric method

in terms of gammas per gram. Use the following formulas:

$$FePO_4 \times 0.3702 = Fe$$

$$FePO_4 \times 0.5292 = Fe_2O_3$$

Calcium

Calcium Oxalate Precipitation. To the filtrate from the mixed phosphates, acidified with *acetic acid*, add an excess of *ammonium oxalate solution*, heat to 50°, and allow to stand overnight in a warm place. Filter on an ashless paper suitable for retaining the fine precipitate, wash with hot water, ignite in a platinum crucible, raising the heat to full redness, and finally over the blast lamp or in a muffle furnace at a higher heat sufficient to convert the calcium carbonate into calcium oxide. Cool in a desiccator over fresh sulfuric acid and weigh.

Calculation. Express result as calcium (Ca) or calcium oxide (CaO); $CaO \times 0.7147 = Ca$.

Magnesium

Ammonium Magnesium Phosphate Precipitation. Add to the calcium oxalate filtrate, with stirring, *ammonium hydroxide* to alkaline reaction, then add an additional portion equal to one-tenth the final volume. Let stand at least 3 hours, filter on a Gooch crucible, wash the ammonium magnesium phosphate with *4% ammonium hydroxide*, ignite at full redness, and weigh the resultant magnesium pyrophosphate.

Calculation. If addition of a little *diammonium phosphate* to the filtrate shows that it is free from magnesium, calculate the weight of magnesium (Mg) or magnesium oxide (MgO) from the magnesium pyrophosphate. Use the following equations:

$$Mg_2P_2O_7 \times 0.2185 = Mg$$

$$Mg_2P_2O_7 \times 0.3623 = MgO$$

(*a*) In case the test of the filtrate shows the presence of magnesium, add more *diammonium phosphate solution*, let stand 3 hours, and filter on the same Gooch crucible on top of the former precipitate, ignite, weigh, and calculate as before. This second operation is not, however, all lost time, as the first precipitate contains all the phosphoric acid in the liquid which, calculated by the factor 0.64 and added to that in the iron and aluminum phosphate, obviates the necessity of a separate determination of total phosphoric acid on a new portion; at least it serves as a check.

If, however, the quantity of ash suffices for a determination of phosphorus on a separate portion, it is advisable to add to the filtrate from the calcium oxalate sufficient *diammonium phosphate solution*, with the phosphoric acid present in the liquid, to precipitate all the magnesium in one operation.

(*b*) A separate determination of phosphorus may be avoided in the first case (excess of P_2O_5 after precipitation with ammonium hydroxide), if the phosphoric acid in excess of that combined with the magnesium is determined in the filtrate, the total phosphorus being the sum of that present in three precipitates, two of magnesium pyrophosphate and one of iron and aluminum phosphates. Whichever course is followed, the added magnesia or phosphoric acid must be removed if sulfuric acid, potash, and soda are to be determined on this portion; this is not recommended unless there is a deficiency of ash.

Chlorine

Silver Chloride Precipitation. To 1 g. of the ash (portion B), add cold *1 + 1 nitric acid*, stir well, filter, and wash, reserving the filter and contents. To the filtrate, add *silver nitrate solution*, and heat nearly to boiling with constant stirring to aid flocculation.

Collect the silver chloride on a porcelain Gooch crucible, wash with hot water, dry cautiously, ignite below redness, and weigh.

Calculation. Use the equation

$$AgCl \times 0.2474 = Cl$$

Also calculate from the chlorine the oxygen equivalent to chlorine by the factor 0.2256. The oxygen equivalent must be deducted from the summation of the analysis, since the bases combined as chlorides do not exist in the oxygenated form.

A method for the direct determination of chlorine is given below.

Sulfur

Solution. If the supply of the ash permits, weigh out a fresh portion of 1 g. (portion C), dissolve in *hydrochloric acid,* filter, wash, and proceed as directed in the second paragraph below; otherwise, proceed as follows.

Silver Chloride Precipitation. Heat nearly to boiling the filtrate from the silver chloride, add *hydrochloric acid* more than sufficient to combine with the silver nitrate, and stir to help coagulate the precipitate. Filter on a fresh paper, wash with water, and reject the precipitate. Run the hot filtrate through the filter, containing the insoluble matter from the solution of the ash, into a porcelain dish, and wash.

Barium Sulfate Precipitation. Evaporate the filtrate to dryness, ignite below redness, add *hydrochloric acid,* and separate silica as in the solution of portion A of the ash. Filter on the paper previously twice used into a platinum dish. Evaporate the filtrate to small volume, take up in hot water, and transfer to a beaker, diluting to 50 or 75 ml. Heat to boiling, add *barium chloride solution* dropwise in slight excess, and allow to stand overnight in a warm place. Filter on ashless paper of close texture, ignite in a platinum crucible, and weigh as barium sulfate.

Calculation. Use the following equations:

$$BaSO_4 \times 0.1374 = S$$

$$BaSO_4 \times 0.3430 = SO_3$$

The direct determination of sulfur in the sample is described below.

Potassium and Sodium

Barium Precipitation. Heat the filtrate from the barium sulfate to boiling, add *barium hydroxide solution* until no more precipitate forms, as seen in the clear supernatant liquid, filter, and wash the precipitate, containing the iron, aluminum, calcium, magnesium, and phosphorus, with hot water.

Ammonium Carbonate Precipitation. Heat the filtrate to boiling, add *ammonium carbonate solution* until a precipitate no longer forms, filter, wash with hot water, and reject the filter and contents. Evaporate the filtrate in a platinum dish to full dryness on the steam bath and ignite below redness to remove ammonium salts. Take up the residue in a little hot water and test with a few drops of *ammonium carbonate solution.*

Joint Chlorides Separation. If, as is usual, the precipitate is slight, filter through a small paper into a small weighed dish, evaporate the filtrate to dryness, ignite below redness until all ammonium salts are removed, cool in an efficient desiccator, and weigh the joint chlorides ($KCl + NaCl$). In order to secure a more exact weighing, heat for a moment, cool in the desiccator, and, with the weights of the former weighing on the pan, weigh rapidly.

If, however, a considerable precipitate forms on testing with *ammonium carbonate,* filter, evaporate the filtrate, ignite, take up in water, and repeat the successive precipitations with *barium hydroxide solution* and *ammonium carbonate solution,* avoiding a large excess of both reagents; filter finally

through a small paper into a small weighed dish and proceed as above.

Potassium Platinochloride Precipitation. Take up the joint chlorides with 50 ml. of water, add *10% chloroplatinic acid solution* (platinum solution) sufficient to combine with both the potash and the soda (5 to 10 ml.), and evaporate to near dryness, moving the dish during the final stages until alkali platinochlorides form a moist deposit. Treat with successive portions of *80% ethanol*, stirring thoroughly with each and allowing to stand 5 to 10 minutes before decanting on a Gooch crucible. Transfer the potassium platinochloride to the crucible, wash with *80% ethanol*, dry for an hour in a boiling water oven, cool, and weigh.

Calculations. Obtain the corresponding weights of K and of K_2O; also calculate the corresponding weight of KCl [1] and subtract from the weight of the joint chlorides, thus obtaining the weight of NaCl from which in turn the weights of Na and of Na_2O may be obtained.

$$K_2PtCl_6 \times 0.1609 = K$$

$$K_2PtCl_6 \times 0.1938 = K_2O$$

$$K_2PtCl_6 \times 0.3067 = KCl$$

$$(KCl + NaCl) - KCl = NaCl$$

$$NaCl \times 0.3934 = Na$$

$$NaCl \times 0.5303 = Na_2O$$

If only determination of the two alkalies together is desired, dissolve the ash in *10% hydrochloric acid*, evaporate to small volume to remove nearly all the hydrochloric acid, dilute without filtering, precipitate with *barium hydroxide solution*, and proceed as above, omitting all details given under chlorine and sulfuric acid.

If only potassium is desired, omit weighing the joint chlorides.

NOTE. The determination of potassium and sodium completes the analysis of the ash. The chlorine and the sulfur, for greater accuracy, are determined directly on new portions of the vegetable or animal substances, incinerated with reagents that prevent the loss sustained in ordinary incineration, as described below.

Calculation of Ash Analysis

The results on water, carbon dioxide, carbon, and sand are not significant and serve chiefly to make the analysis complete. A summation reasonably close to 100 furnishes a certain degree of assurance of the accuracy of the work, although it may not detect an erroneous separation of two constituents such as potash and soda. Unlike water, carbon dioxide, and carbon, the sand is a true constituent of the product on the market, although not of the clean product protected from dust and spattering during growth. It is, however, the analysis calculated free from all these extraneous constituents, including sand, that has scientific value.

To obtain the percentage of this "pure ash," divide 100 by the sum of the percentages of the significant constituents and multiply the percentage of "crude ash" in the sample by this factor; to obtain the percentage of each significant constituent in the pure ash, multiply the percentage of each by the same factor. The percentage of sand, if excluded from the analysis, should be separately given, since the sum of this and the pure ash constitute the actual ash which should figure in the summation in obtaining the nitrogen-free extract by difference.

The percentage of pure ash in the analysis thus calculated is not scientifically perfect, as the sulfur and chlorine present in the crude ash, owing to loss during burning, do not represent the full amounts present in the product. If greater accuracy is desired, the sulfur and chlorine should be determined separately, in fresh portions of the material,

by methods described below. Introducing these corrected figures in the analysis necessitates considerable calculation which can be obviated if percentages of all the constituents are calculated to the original unburned sample.

To carry out this plan, multiply the percentage of each significant constituent, excluding chlorine and sulfur dioxide, of the ash analysis by the percentage of ash in the sample and divide by 100; to the list of percentages thus calculated, add those of chlorine and sulfuric acid (SO_3) separately determined. The analysis on this basis does not correspond exactly with the percentage of either crude or pure ash, but is comparable with the results of determinations of the minor mineral constituents, which customarily are calculated directly to the sample, although in gammas per gram.

Some analysts express all results in terms of percentages of the elements in the unburned material either on the original or water-free basis.

Procedure with One Gram of Ash. If the entire analysis must be made on one portion of 1 g. (or 0.5 g.), determine the constituents in the following order: *water*, *carbon dioxide*, *chlorine*, *carbon*, *sand*, *silicon*, *iron*, *aluminum*, *calcium*, *magnesium*, *phosphorus*, *sulfur*, *potassium*, and *sodium*. This involves (1) the use of nitric acid in the determination of carbon dioxide, (2) the removal of the excess of silver nitrate, after precipitation of the chlorine, by precipitation with hydrochloric acid, (3) the evaporation in porcelain to remove the nitric acid, (4) the precipitation of iron and aluminum phosphates, (5) the skillful handling of the magnesium and phosphoric acid, first by precipitation of the magnesium by part of the phosphoric acid, followed by the precipitation of the remainder of the phosphoric acid with added magnesia mixture, (6) the precipitation of the sulfuric acid with barium chloride, (7) the removal of the added magnesium and barium by precipitation with ammonium carbonate, and finally (8) the determination of potassium and sodium in the usual manner.

It also assumes that the phosphoric acid is in excess of the iron, aluminum, and magnesium, which is usually true of cereal and other seed ashes. If not in excess, as in certain leaf foods, phosphoric acid must be added as diammonium phosphate and the excess removed with barium hydroxide, and this in turn with ammonium carbonate, followed by determination of the alkalies. The general principle to be observed in making such an extended and intricate analysis would be to avoid adding a reagent containing an element present in the ash (chlorine, magnesium, phosphorus, potassium, and sodium), until after that constituent had been separated and determined, to remove the excess of each reagent, and to avoid addition of acids, other than hydrochloric, carbonic, and acetic, which alone and in combination cannot be removed by heating.

Sulfur

(Direct Determination)

As noted above, the ordinary method of preparing the ash causes a loss of the sulfur present in the sample and a gain of sulfur from the products of combustion. The Carius closed tube method,[2] involving heating with fuming nitric acid in a closed tube, and the Fraps nitric acid-potassium nitrate fusion method [3] were shown by Thatcher [4] to yield lower results than the Osborne peroxide method.[5]

Osborne Peroxide Method.[5] Although originally designed for use in the ultimate analysis of proteins, this method is equally well suited for the direct determination of sulfur in vegetable and animal foods in which part of the sulfur may be in forms other than a constituent of the protein molecules.

Process. Weigh into a nickel crucible 10 g. of *sodium peroxide* and make into a paste with a little water. Heat cautiously with an alcohol flame until foaming subsides, then cool somewhat and add 1 to 2 g. of the air-dry sample, stirring with a nickel rod. Raise the heat cautiously, continuing the stirring to

avoid loss by frothing, and add from time to time small portions of *sodium peroxide* until the oxidation is complete. Cool, boil the melt with water and *hydrochloric acid* until all sulfates are in solution, filter, evaporate the filtrate to dryness, separate silica with a few drops of *hydrochloric acid*, and proceed with the determination of sulfuric acid as barium sulfate as described in the scheme above.

Calculate as sulfur (S) or as sulfur trioxide (SO_3).

Skinner Peroxide Method.[6] This process in essentially the form here described has been official with the A.O.A.C. since 1906.

Process. Make 1 to 2 g. of the air-dry sample into a paste with 2 ml. of water in a nickel crucible, add 5 g. of *anhydrous sodium carbonate* and mix with a nickel rod. Add about 10 portions of 0.5 g. each of *sodium peroxide*, stirring after each addition, until the mixture is nearly dry and granular. Heat cautiously to fusion over an alcohol flame, avoiding ignition which is disastrous. Cool somewhat, cover the melt with a 0.5-g. layer of *sodium peroxide* and heat gradually to complete fusion, continuing for 10 minutes with gentle rotation to wash down the sides. Cool, place the crucible and contents in a beaker, add 100 ml. of water, then *hydrochloric acid* to slightly acid reaction, boil, filter, separate the silica, and determine the sulfuric acid as barium sulfate as described above.

Frear Copper Nitrate Gravimetric Method.[7] This application to plant material of the Benedict-Denis reagent, used in the determination of sulfur in urine,[8] was developed at the Rhode Island Agricultural Experiment Station.

Reagent. *Benedict-Denis Reagent.* Dissolve 25 g. of $Cu(NO_3)_2 \cdot 3H_2O$, 25 g. of NaCl, and 10 g. of NH_4NO_3 in water and dilute to 100 ml.

Process. *Incineration.* Weigh 1 g. of the finely ground sample into an 8-cm. porcelain evaporating dish, add 20 ml. of *Benedict-Denis reagent*, mix, heat slowly almost to boiling, and evaporate (1.5 hours) at a temperature of 95 to 100°, adding water from time to time to infuse the sample with the reagent. When dry, ignite at dull redness for 10 minutes in an electric furnace or over a sulfur-free flame.

Solution and Precipitation. Cool, add about 50 ml. of *6 N hydrochloric acid*, dissolve the residue with heat, filter to remove the unburned carbon, and wash the filter with hot water. Dilute the filtrate to 350 ml., heat to boiling, add 10 to 20 ml. of *10% barium chloride solution*, allow to stand 18 hours, collect the barium sulfate on a filter, and determine in the usual way.

Conduct for each run a blank determination with only the reagents.

Chlorine

(Direct Determination)

Davies Sodium Carbonate-Silver Chloride Gravimetric Method.[9] Process. To avoid loss of chlorine, the material is ashed with sodium carbonate.

Incineration. Weigh into a platinum dish a suitable quantity of the material, which, for substances difficult to sample (such as meat) or containing but a small amount of chlorine (such as flour), should not be less than 5 to 10 g. Add *chlorine-free sodium carbonate* equivalent to at least 5% of the dry matter, mix thoroughly, and burn at a heat below bright redness in the usual manner until most of the carbon has been removed.

Silver Chloride Precipitation. Treat the ash with *dilute nitric acid*, aliquoting if the quantity of chlorine is large, and proceed as directed for the general scheme above.

Volhard Ferric Alum Volumetric Method Modified by Rothmund and Burgstaller.[10] This valuable method was devised at Prague University.

Process. *Incineration.* Burn with *so-*

dium carbonate and dissolve the ash in *nitric acid* as directed for the Davies Method above.

Silver Chloride Precipitation: Add to the solution *0.1 N silver nitrate solution* in excess of that required to precipitate the chlorine and heat until the precipitate of silver chloride coagulates completely.

Titration. Cool, add 5 ml. of *saturated ferric alum solution* as indicator, and titrate back with *0.1 N ammonium thiocyanate solution* until the brown color of ferric thiocyanate appears.

CALCULATION. Obtain the weight of chlorine (W) by the following formula:

$$W = 0.003546 \times (A - T)$$

in which A is the number of milliliters of 0.1N $AgNO_3$ and T is the number of milli-

NOTES. The coagulation by heat is a feature introduced by Rothmund and Burgstaller to obviate the fading of the color when small amounts of chlorine are present.

A sharp color change may also be secured, as recommended by Drechsel,[11] by filtering from the precipitated silver chloride and titrating the filtrate. To avoid washing the precipitate, make up to 100 ml. in a volumetric flask, filter through a dry paper, and titrate 50 ml. of the filtrate.

Mohr Chromate Volumetric Method. This method can be used only when the solution is neutral. Evaporate to dryness the nitric acid solution, obtained as above directed, and heat further until all free acid is removed. Dissolve the residue in water and titrate directly with the *standard 0.1 N silver nitrate solution*, using *5% potassium chromate solution* as indicator; 1 ml. of 0.1 N $AgNO_3$ = 0.003546 g. of Cl.

PHOSPHORUS

(Direct Determination)

Phosphorus, whether or not in the form of phosphoric acid, is the chief acid-forming mineral constituent present in cereals, oil seeds, leguminous seeds, and meat. In milk and fruits, also in root, tuber, and leaf vegetables, the phosphorus is not present in sufficient amount to combine with all the basic mineral constituents and as a consequence these latter combine in part with the carbonic acid formed during burning, yielding an alkaline ash.

Sonnenschein Molybdate-Magnesium Pyrophosphate Gravimetric Method.[12] The method is direct in that phosphoric acid is determined directly in the weighed and incinerated portion of the sample without weighing the ash; it is, however, indirect in the sense that the phosphoric acid is first precipitated as phosphomolybdate and not directly as ammonium magnesium phosphate. The method as given herewith is essentially that which was adopted in 1884 [13] and is still the official method.

REAGENTS. *Magnesium Nitrate Solution.* Dissolve 15 g. of MgO in a slight excess of 1 + 1 HNO_3, then add MgO in excess, boil, filter, and dilute to 100 ml.

Molybdate Solution. Dissolve 100 g. of $H_2MoO_4 \cdot H_2O$ in a mixture of 144 ml. of NH_4OH and 271 ml. of water, then pour slowly with stirring into a mixture of 489 ml. of HNO_3 and 1148 ml. of water. Allow to stand in a warm place for several days and decant off the clear liquid.

Magnesia Mixture. Dissolve 11 g. of crystalline $MgCl_2 \cdot 6H_2O$ and 28 g. of NH_4Cl in 130 ml. of water. Add 27 ml. of NH_4OH and dilute to 200 ml. Let stand at least 1 day and decant for use. Use 10 ml. for each 0.10 g. of P_2O_5.

PROCESS. *Incineration and Solution.* Moisten 5 or 10 g. of the air-dry sample in a platinum or porcelain dish with 10 ml. of *magnesium nitrate solution*, dry, ignite, and boil the residue with *10% hydrochloric acid*. Filter, wash, add *ammonium hydroxide* until a precipitate just forms, then add a few drops of *nitric acid* to dissolve the precipitate and

about 15 g. of *crystalline ammonium nitrate.* Stir until the nitrate dissolves and heat the solution to boiling.

Precipitation as Phosphomolybdate. Add to the solution an excess of *molybdate solution* (about 70 ml. for each 0.1 of phosphoric acid), test while still hot with a few milliliters of *molybdate solution,* adding more if necessary, allow the precipitate of ammonium phosphomolybdate to settle 1 hour, filter, and wash with cold water.

Precipitation as Magnesium Pyrophosphate. Dissolve the precipitate from the paper into a beaker with *1 + 9 ammonium hydroxide,* and wash with hot water. Nearly neutralize with *hydrochloric acid,* cool, and add an excess of *magnesia mixture,* drop by drop with stirring, then *ammonium hydroxide* to one-tenth the final volume. Filter after 3 hours on a Gooch crucible, wash with *1 + 9 ammonium hydroxide solution,* dry, ignite to full redness for 5 minutes, cool, and weigh.

CALCULATION. Employ the following formulas:

$$Mg_2P_2O_7 \times 0.2784 = P$$

$$Mg_2P_2O_7 \times 0.6379 = P_2O_5$$

$$P_2O_5 \times 0.4365 = P$$

Zinzadze Molybdenum Blue Colorimetric Method.[14] The color formed by this method is more stable than that by the Osmond-Zinzadze molybdenum trioxide reduction method, but is formed more slowly. It yields good results in the presence of silica, arsenic, iron, and nitrate. Zinzadze, formerly of Tiflis University, Georgia (Caucasus), published from the New Jersey Experiment Station. See also Arsenic.

APPARATUS. *Colorimeter.*

REAGENTS. *α-Dinitrophenol (2,4-dinitrophenol) Solution,* saturated at 50°. Let settle overnight and decant.

Molybdenum Blue Reagent. The somewhat tedious method of preparation is described in Zinzadze's paper. Reputable chemical houses supply the reagent with a guarantee of acidity (10 ml. of a 1% dilution = 24.9 to 25.1 ml. of 0.1 *N* NaOH solution) and molybdenum concentration (5 ml. = 4.9 to 5 ml. of 0.1*N* potassium permanganate solution).

PROCESS. *Acid Adjustment.* Pipet into a 50-ml. volumetric flask, with a mark at 30 ml., 0.5 to 15 ml. of the standard or the unknown, containing 0.01 to 0.3 mg. of phosphorus pentoxide, and 5 drops of *α-dinitrophenol indicator.* Neutralize with *2% sodium bicarbonate solution* or *1.0 N sulfuric acid* to a faint yellow color. Add 5 ml. of the *acid* and 5 ml. of *8% sodium bisulfite solution,* make up to about 30 ml., and shake well.

Color Formation. After allowing to stand overnight or heating 1 hour on the steam bath, add 5 ml. of a ten-fold dilution of *molybdenum blue reagent.* Heat at least 30 minutes and shake thoroughly. Allow to cool to room temperature, dilute to the mark, and shake thoroughly.

Color Measurement. Compare the color of the unknown with that of a *standard solution* treated in like manner in an ordinary colorimeter or measure the color intensity in a photoelectric colorimeter.

NOTE. The velocity of the formation of the blue color is a function of temperature. The maximum color at 20 to 30° is attained in 3 days, at 50° in 10 hours, at 70° in 3 hours, at 95 to 100° in 30 minutes, and by boiling in 4 to 5 minutes. The color is stable for 2 to 3 days if kept stoppered in the dark. With pure reagents a blank remains slightly yellowish.

Osmond-Zinzadze Molybdenum Trioxide Reduction Colorimetric Method.[15] APPARATUS. *Colorimeter.*

REAGENTS. *Molybdenum Trioxide Reagent.* To 101 ml. of 25 *N* H_2SO_4 in a 500-ml. Erlenmeyer flask, add molybdic acid (anhydride) containing exactly 4.01 g. of molybdic trioxide, dissolve by very gentle boiling with occasional shaking, avoid the

evolution of white fumes, cool to room temperature, and pour slowly into a 1-liter flask containing about 900 ml. of water. Dilute to about 988 ml., cool further, and make up to the mark. Test by diluting 10 ml. delivered from a wetted pipet to 100 ml. and titrate with 0.1 *N* NaOH solution (phenolphthalein) of which 24.9 to 25.1 ml. should be required.

Gum Arabic Solution. Dissolve 10 g. of the pure gum arabic in 1 liter of water at about 50° by shaking for 30 minutes or until clear. Cool to room temperature and add 1 ml. of toluene.

Stannous Chloride Solution. Add 0.16 g. of fresh $SnCl_2 \cdot 2H_2O$ to 200 ml. of 1% gum arabic solution and shake well. Ignore a slight turbidity that disappears during the determination. The solution keeps only 12 hours.

Process. *Reduction.* Treat an aliquot of the unknown and the standard as in the Zinzadze molybdenum blue method up to the addition of the molybdenum blue reagent.

Color Formation. Cool, add 5 ml. of the *molybdenum trioxide reagent,* and mix well, then add rapidly with shaking 5 ml. of *stannous chloride solution,* dilute to 50 ml., and shake thoroughly. Treat the standard simultaneously in the same manner as the unknown.

Color Comparison. After allowing to stand 20 minutes, make the comparison. After 6 hours, the color fades.

Pemberton Molybdate Volumetric Method. As originally proposed by Pemberton, the method had certain defects which were overcome by the following modification which has long been official.

Kilgore Modification.[16] Reagents. *Molybdate Solution.* To 100 ml. of molybdate solution, prepared as directed under the Gravimetric Method above, add 5 ml. of HNO_3 and filter immediately before using.

Standard Sodium Hydroxide Solution. Dilute 323.81 ml. of the 1.0 *N* solution, free from carbonates, to 1 liter; 1 ml. = 1 mg. or 1% of P_2O_5 on the basis of 0.1 g. of the sample.

Standard Acid. Dilute HCl or HNO_3 so that 1 ml. corresponds to 1 ml. of the standard alkali.

Process. *Solution.* See Molybdate Gravimetric Method above.

Molybdate Precipitation. Remove an aliquot corresponding to 0.4 g. of the sample to a beaker and add 5 or 10 ml. of *nitric acid* (or an equivalent amount of ammonium nitrate solution), according as the solvent was nitric or hydrochloric acid. Add *ammonium hydroxide* until a precipitate forms that dissolves slowly on vigorous stirring. Dilute to 75 to 100 ml. and adjust to 25 to 30°. Add 20 to 25 ml. of freshly filtered *molybdate solution* and agitate in a mechanical shaker or stirrer for 30 minutes at room temperature. Decant at once through a filter and wash the precipitate twice by decantation with 25- to 30-ml. portions of water, mixing well and allowing to settle after each addition. Transfer the precipitate to the filter and wash with cold water until the filtrate from 2 fillings yields a pink color with *phenolphthalein* and 1 drop of *standard alkali.*

Titration. Return the precipitate, together with the filter, to the beaker, dissolve in a small excess of *standard alkali,* add a few drops of *1% phenolphthalein in ethanol,* and titrate with *standard acid.*

Calculation. Use the following: 1 ml. of the standard sodium hydroxide solution = 1 mg. or 0.25% of P_2O_5.

Potassium

Schlössing-Wense Perchlorate Gravimetric Method.[17] The method depends on the insolubility of potassium perchlorate and the solubility of the corresponding sodium salt in 97% ethanol. The advantage over the standard platinochloride method of cheapness of the reagent is offset by the labor in-

volved in its purification and the danger of explosions if evaporation with ethanol is carried out near a free flame.

REAGENTS. *Perchloric Acid Solution*, 20%, of highest purity.

Wash Liquid. Prepare a 0.2% solution of $HClO_4$ in 97% ethanol and saturate with $KClO_4$.

PROCESS. *Determination of Joint Chlorides.* See Platinochloride Method under Scheme for Complete Ash Analysis, Potassium and Sodium, above.

Perchloric Acid Treatment. Dissolve the joint chlorides, containing not more than 0.5 g. of potassium salt, in 20 ml. of hot water and add 5 ml. of *20% perchloric acid* (sp.gr. 1.12) and evaporate carefully until salts separate. Take up in 10 ml. of hot water, add 5 ml. of *perchloric acid*, then evaporate to dryness on the water bath and finally on the sand bath. Repeat if necessary the addition of water and *perchloric acid* until heavy fumes of the acid appear during evaporation.

Ethanol-Perchloric Acid Digestion. Cool below room temperature, add 20 ml. of the *wash liquid*, and stir for a time to dissolve the sodium perchlorate. Crush the precipitated potassium perchlorate and let stand 30 minutes in an ice-water bath. Decant through a tared Gooch crucible, wash three times with small portions of the *wash liquid*, and, if the amount of precipitate is small, dry at 130° and weigh.

If the amount of precipitate is large, redissolve in a little hot water, add 1 ml. of *perchloric acid*, evaporate, and treat as before. Finally transfer the precipitate to the crucible, wash with small portions of ice-cold *wash liquid*, dry at 130° for 1 hour, and weigh.

CALCULATION. Employ the following formulas:

$$KClO_4 \times 0.2822 = K$$

$$KClO_4 \times 0.5381 = KCl$$

$$KClO_4 \times 0.3399 = K_2O$$

Smith and Ross Modification.[18] Instead of 97% ethanol, a mixture of equal volumes of *n-butanol* and *ethyl acetate* is used for the digestion and washing. The final drying is at 350° in a muffle.

Cobaltinitrite Gravimetric Method. See Part II, D2.

Cobaltinitrite Volumetric Method.[19]

SCHEME FOR TWELVE INORGANIC ELEMENTS

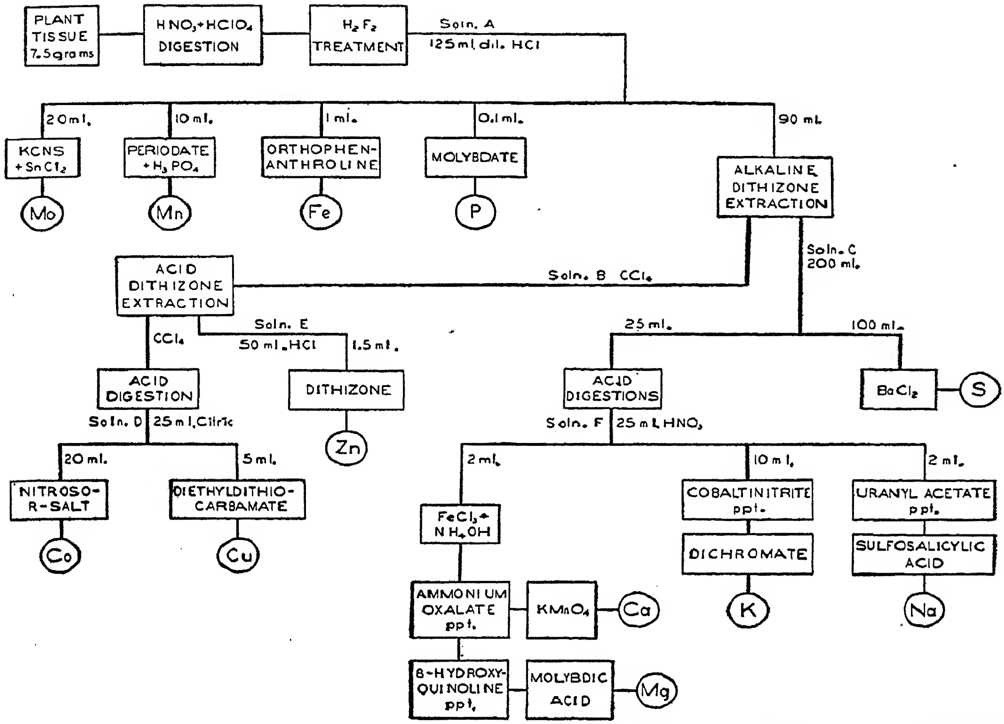
The scheme (Fig. 59) is from a recent paper by Parks, Hood, Hurwitz, and Ellis,[20] of the U. S. Plant, Soil, and Nutrition Laboratory, Agr. Res. Adm., Ithaca, N. Y. In addition to the principal elements, several trace elements are included.

b. Minor Inorganic Elements

The results are expressed in terms of gamma per gram (γ/g.) which is equivalent to milligrams per kilo (mg./k.) and parts per million (p.p.m.).

The so-called trace elements include such heavy metals and other elements as occur in minute amounts in foods through normal processes of growth, through accidental contamination, or through willful addition. Of these, two, iron and aluminum, often due to adhering soil, are determined as the mixed oxides in the ordinary course of ash analysis, but not with the degree of accuracy as by modern micro or semi-micro methods. The heavy metals, iron, manganese, copper, zinc, and perhaps others, in small but appreciable amounts, are normal constituents of foods of more or less significance in nutrition. The addition of copper in the form of sulfate for "greening" canned vegetables and pickling in brass or copper utensils was discontinued because of legal restrictions, and the menace of lead from water pipes disappeared with the substitution of iron for lead in plumbing.

The metals most commonly derived from utensils and containers are iron, copper, zinc,



Courtesy of Ind. Eng. Chem., Anal. Ed. 1943, **15,** *529*

G. 59. Parks, Hood, Hurwitz, and Ellis Scheme for Twelve Inorganic Elements.

tin, and lead; those from insecticides and fungicides in the so-called spray residues are copper, lead, arsenic, and fluorine. The last three occur naturally only in insignificant traces in foods.

Iodine is present in minute but determinable amount in salt water algae and salt marsh plants, also in animal sea food. Iodized salt (0.02% KI) is sold as a preventive of goiter. Bromine also occurs in minute amount in marine plants and animals and is a constituent of flour improvers and yeast foods.

Fluorine, although occurring naturally in the enamel of teeth, in excessive amount causes a mottled appearance, but recent investigation indicates that such teeth are more resistant to decay than those with lower fluorine content.

Boron recently has been shown to be beneficial to plants and animals; on the other hand, selenium has a toxic action, as shown by the mortality of cattle in certain regions where its content in forage plants such as "loco weeds" of the genus *Astragalus* is abnormally high.

Methods. The methods described for the most part are not those used in metallurgical and mineralogical analysis, the allowable errors of which often exceed the total amount in foods, nor have many of them been inherited from the toxicologist, but are indi-

vidual micro methods developed in agricultural, biological, nutritional, and regulatory laboratories. Comprehensive analytical schemes are lacking.

Although spectroscopes have long served for the qualitative detection of the elements, spectroscopic methods, especially those employing the spectrograph, are of more recent introduction and application in food analysis is still more recent. The reader is referred to the work of Brode [21] and of Thompson [22] for an introduction to the subject. The high cost of the instrument precludes its general use.

Aluminum

Although aluminum is a natural constituent of foods, that found on tubers, roots, fruits, vegetables, and spices may be due partly or largely to adhering soil, whether from direct contact, spattering, or dust clouds.

Gravimetric Alumina Method. In the complete analysis of the ash, alumina is usually included with iron as "iron oxide and alumina" or merely "iron oxide," since alumina is often present in so small amount as not to be significant in the second place of decimals when the results are calculated as percentages on the fresh basis. If, however, the weight of mixed iron and aluminum phosphates is sufficient, the iron oxide may be determined by permanganate titration, the phosphoric acid by the molybdate method, and the alumina obtained by difference, subtracting the weight of the other two from the total weight. At best, such a procedure throws the analytical errors of three determinations on the alumina. (See Analytical Scheme, Part I, C8*a*.)

Fortunately colorimetric methods are available for the micro determination of aluminum (whether calculated as the element or the oxide) either directly in a solution of the ash or in the mixed phosphates.

Atack Alizarin Red S Colorimetric Method.[23] REAGENTS. *Alizarin Red S (Monosodium Alizarin Sulfonate) Solution*, 1%.

Standard Aluminum Solution. See following methods.

PROCESS. *Incineration and Solution.* Burn 2 to 20 g. of the sample to a white or gray ash in the usual manner. Although aluminum salts are not volatile, care should be exercised to avoid fusion of the ash and consequent difficulty of solution. Add *nitric acid* during the ignition if the carbon resists ordinary treatment. Heat for a time with *1 + 1 hydrochloric or concentrated sulfuric acid* in platinum to insure complete solution.

Separate silica, as described under Complete Ash Analysis (Part I, C8*a*) if present in considerable amount.

Color Comparison. To the acid solution, measuring 10 to 20 ml., add 10 ml. of *glycerol*, 5 ml. of *1% alizarin red S solution*, and dilute to 40 ml. Make slightly alkaline with *ammonium hydroxide* and allow to stand 5 minutes. Add *dilute acetic acid* to acid reaction, as shown by the complete color change of the alizarin red S, and dilute to 50 ml. Compare without delay with a *standard aluminum solution* diluted to an aluminum content approximately that shown by the color of the unknown.

NOTE. Underhill and Peterman [24] (Yale University) direct as follows: Treat the initial acid solution in 5-ml. portions in separatory funnels with 0.1 to 1 ml. of *60% ammonium sulfocyanate* and shake out the iron sulfocyanate with *ether*. To the iron-free solution, add 2.5 ml. of *glycerol-citric acid mixture* (4 parts of glycerol to 1 part of 10% citric acid) and 1 drop of *0.5% alizarin red S*, and compare with a standard solution as in the original method.

Hammett and Sottery Aluminon Colorimetric Method. The dye aurin tricarboxylic acid (No. 557, Schultz: *Farbstofftabellen*, Berlin, 1923) was proposed by Hammett and

Sottery [25] (Columbia University) as a specific reagent in testing for aluminum because of the bright red lake it forms with that ion, which, although soluble in ammonium hydroxide, whenever formed in the presence of an acetic acid acetate buffer does not immediately decompose, whereas the chromium lake is at once decolorized. Most of the other heavy metals give white or no precipitates; the alkali earths, however, give red and ferric salts violet precipitates. Alkali earths are readily removed by precipitation of iron and aluminum as basic acetates, and the latter is separated from the former by reason of its solubility in sodium hydroxide solution.

I. Myers, Mull, and Morrison Modification.[26] An adaptation of the Hammett and Sottery method to animal tissues, devised at Western Reserve University.

Apparatus. *Color Comparison Tubes.*

Reagents. *Aluminon Reagent* (ammonium salt of aurin tricarboxylic acid). Dissolve 1 g. of aurin tricarboxylic acid (Eastman) in 200 ml. of water, add 2.5 ml. of NH_4OH, and dilute to 1 liter. Keep in the dark.

Ammonium Carbonate Mixture. Dissolve 180 g. of ammonium carbonate in 420 ml. of NH_4OH and 1700 ml. of water.

Sodium Hydroxide, 6 *N*. Prepare from aluminum-free sodium.

Standard Aluminum Solution. Dissolve 3.518 g. of potash alum, five times recrystallized and dried over $CaCl_2$ and make up to 1 liter. Dilute 50 ml. to 1 liter; 1 ml. = 0.01 mg. of aluminum.

Process. *Wet Combustion.* Mix a suitable amount (2 to 20 g.) of the sample in a flask with sufficient *sulfuric acid* and *60% perchloric acid,* in the ratio of 1 + 1.25, to form a semi-liquid mass. Add a few silica chips and heat at first with a low flame, gradually increasing the heat as the combustion proceeds. The charred mass at first formed gives place to a thick brown liquid that gradually clears and finally becomes water-clear with a white precipitate. Concentrate to 5 ml.

Coprecipitation of Iron and Aluminum. Transfer the digested product, including the precipitate which is partially dissolved in hydrochloric acid, to a volumetric flask, and make up to volume. Pipet the whole solution (if only a little aluminum is suspected) into a 50-ml. centrifuge tube, add 1 mg. of *ferric iron* (if the amount of iron is small), and make up to 15 ml. with water. Neutralize the solution with *ammonium hydroxide,* using 1 drop of *very dilute methyl red solution* as indicator. Add 1 ml. of *saturated ammonium acetate solution,* keep in boiling water for 10 minutes, allow the precipitate to settle, centrifuge, decant off, and discard the supernatant liquid.

Separation of Aluminum from Iron. Dissolve the precipitate in 1 ml. of *6 N hydrochloric acid,* warm if necessary, and dilute to 15 ml. Cool, add 1.25 ml. of *glacial acetic acid* and 5 ml. of *6 N sodium hydroxide solution.* Allow to stand 1 hour, moving the tube so as to wash down the sides, centrifuge, and decant the clear solution into a 50 ml. comparison tube, rejecting the precipitate that contains the iron.

Color Formation. Add 1.1 ml. of *6 N hydrochloric acid* and 0.75 ml. of *glacial acetic acid* to the solution. Make up to 35 to 40 ml., add *aluminon reagent,* in the proportion of 1 ml. for each 0.01 mg. of aluminum, shake, allow to stand 10 minutes, and add 5 ml. of *ammonium carbonate mixture,* which should bring the pH of the solution to 7.0 to 7.3, and again shake.

Color Comparison. Promptly compare with a *standard aluminum solution* containing the same amounts of the reagents and treated in the same manner as the unknown, inverting known and unknown solutions the same number of times to secure equal evolution of carbon dioxide.

II. Winter, Thrun, and Bird Modification.[27] The following directions (Michigan

Experiment Station) apply to vegetable products.

Apparatus. *Duboscq Colorimeter.*

Reagents. See above.

Process. *Color Formation.* Transfer the slightly acid solution to a 50-ml. volumetric flask, dilute to about 20 ml., and add 5 ml. of *5 N ammonium acetate solution*, 5 ml. of *1.5 N hydrochloric acid*, and 2 ml. of *aluminon reagent.* Heat in a water bath at 80° for 10 minutes, add 5 ml. of *5 N ammonium chloride solution*, cool, add 5 ml. of *3.2 N ammonium carbonate solution* with gentle shaking, make up to the mark, and mix.

It is important that the pH at this point be 7.1 to 7.3 and that the red color of a blank disappear in about 15 minutes. The amount of ammonium carbonate necessary to bring to the proper pH should be determined by neutralization of a parallel solution without addition of the aluminon reagent.

Color Comparison. Compare the color of the known and unknown solutions after allowing to stand 20 minutes to decolorize the excess of dye.

Calculation. Winter and associates recommend conducting along with the actual analyses parallel tests on *four standard solutions* containing 0.01 to 0.07 mg. of aluminum in 0.02-mg. increments, and plotting a curve with these values against readings on the Duboscq colorimeter scale as coordinates. On such a curve, once plotted, subsequent results may be read without conducting tests on more than a single standard solution.

Arsenic

Formerly tests for arsenic, whether in foods, drink, or wall paper, were chiefly limited to securing evidence in poison cases, but the present extensive use of arsenical sprays for fruits and vegetables and the adoption of legal limits has greatly extended the need not only for reliable tests but also for quantitative methods. The amount of arsenic in foods under normal conditions is detectable only by delicate spectroscopic method.

Marsh Arsine Reduction Method. The originator, J. Marsh (1790–1846), was an assistant of Faraday. At a red heat, arsine (AsH_3) is decomposed into metallic arsenic and hydrogen.

Apparatus. The *Train* (Fig. 60), devised by Johnson and Chittenden, consists of (1) a U-tube, containing cotton moistened with 10% lead acetate solution to trap hydrogen sulfide, (2) a U-tube filled with calcium chloride, and (3) a hard glass combustion tube drawn out near the end to a narrow constriction and at the end to a narrow opening. To prevent bending in the furnace and to distribute the heat, the latter is wrapped with wire gauze.

Process. *Digestion. A. Johnson-Chittenden-Gautier Procedure.* To 100 g. of the finely ground or chopped sample in a porcelain caserole add 23 ml. of *arsenic-free nitric acid* and heat with stirring on a ring over a dry basin showing an air temperature of 150 to 160°. Remove the heat when the mixture takes on an orange color and add 3 ml. of *arsenic-free sulfuric acid,* stirring during the violent reaction and evolution of fumes. Again heat and, when the temperature reaches 180°, add dropwise 8 ml. of *nitric acid* and heat at 200° until dense sulfuric fumes appear, leaving a dry charred mass in the dish.

Grind the residue, extract with hot water, filter, and evaporate the filtrate to small volume. Dissolve the residue in cold *20% sulfuric acid* and treat by either the Marsh or the Gutzeit methods.

B. Sanger Procedure.[28] Sanger uses a smaller amount of material (5 to 25 g.) and 20 ml. of *sulfuric acid* at the start, repeating the addition of *nitric acid* in 2-ml. portions and heating after each addition until sulfuric acid fumes appear, then heating to remove all nitric acid and diluting. To insure reduction to an arsenious condition, a final evaporation

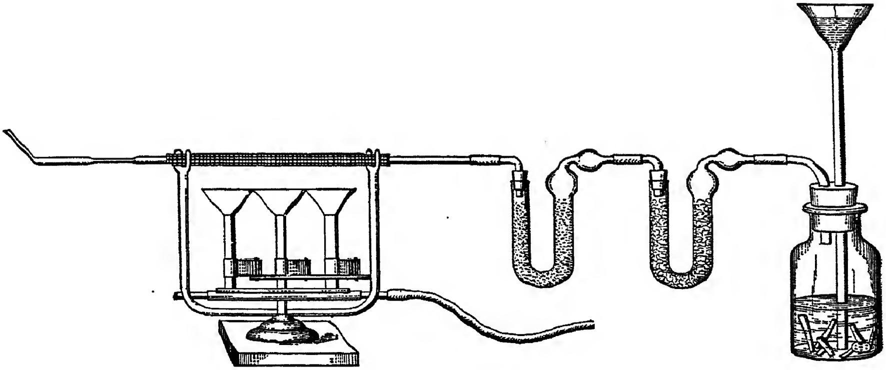


FIG. 60. Marsh Arsenic Apparatus.

with *saturated sulfurous acid solution* is recommended.

Arsine Evolution. Into the generating bottle, introduce 20 to 30 g. of *arsenic-free zinc* and a perforated platinum disk to complete an electric couple. Close the stopper and connect with the lead acetate U-tube, and then add *20% sulfuric acid* through the funnel. When all air has been forced out by the hydrogen, heat the tube to bright redness. If the reagents are arsenic-free, no mirror will form in the constriction.

This having been accomplished, slowly introduce the acid solution of the unknown as above described or, in the case of surface contamination of fruits, by washing with *acid.* As the strength of the acid weakens, add *30%* and later *40% sulfuric acid* until all arsenic has been driven off. An hour's run is sufficient to establish the absence of arsenic. In 2 or 3 hours arsenic will be driven off and condensed at the constriction.

CALCULATION. To determine the weight of arsenic, cut off the portion of the tube containing it, weigh on a delicate balance, then dissolve off the arsenic in *sodium hypochlorite solution,* wash with water, then with *ethanol,* dry, cool, and weigh again, obtaining the weight of the arsenic by difference. Antimony, if present, will not dissolve in the hypochlorite.

NOTE. For small amounts, Sanger compares tests on the unknown with aliquots of a standard solution prepared by dissolving 1 g. of *arsenious oxide* in *sodium hydroxide solution,* acidulating, diluting to 1 liter, and further diluting of 10 ml. of this stock solution to 1 liter.

Gutzeit Silver Nitrate Paper Stain Method. In this, as in the Marsh method, hydrogen is generated in the presence of the arsenic compound, but the arsine, instead of being reduced, reacts with *silver nitrate* impregnated in a strip of paper to produce a coloration of $AsAg_3$.

I. Sanger and Black Mercuric Salt Modification.[29] Mercuric chloride or bromide is substituted for silver nitrate as the impregnating chemical.

APPARATUS. The *Bishop Apparatus* (Fig. 61) consists of a 30-ml. salt-mouth bottle (*A*) to which is attached by a perforated stopper a series of three upright tubes. The lower tube (*B*), 1-cm. bore and 7 cm. long, contains strips of filter paper impregnated with 5% lead acetate solution and dried; the middle tube (*C*), somewhat shorter than the first, is filled loosely with cotton impregnated with 1% lead acetate solution. These two tubes

serve to absorb the liberated hydrogen sulfide. The reaction with the arsine takes place in the upper double-bent tube (*D*) of 2.5-mm. bore, containing a strip of cold-

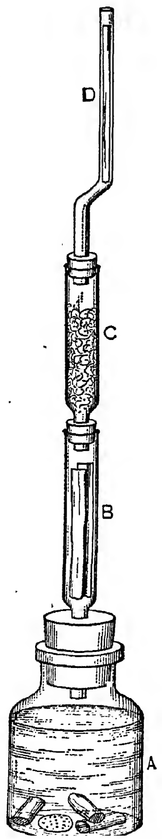


Fig. 61. Bishop Arsenic Apparatus.

pressed drawing paper, 2 mm. wide, rendered sensitive to arsenic by soaking in 5% mercuric chloride or bromide in ethanol and drying. Sanger and Black attribute the nonconcordant results of earlier analysts to uneven penetration of the arsine into filter paper.

Process. *Arsine Evolution.* In the bottle, place 10 g. of *stick zinc*, a small amount of *crystalline stannous chloride*, a perforated platinum disk, and from 2 to 5 g. of the material suspected of containing arsenic, or a solution obtained by digesting the charred mass, as described above, with *20% sulfuric acid.* Nearly fill the bottle with *20% sulfuric acid*, attach the three tubes, and allow to react for 45 minutes.

Color Comparison. Compare the color of the sensitized strip with that of others treated in the same apparatus by the action on 0.005 to 0.05 mg. of *arsenious oxide* added as aliquots of a standard solution.

Notes. In the *Methods of the A.O.A.C.* are given minute instructions for conducting the test.

Remington, Coulson, and von Kolnitz,[30] of the laboratory of the Medical College of the State of South Carolina, shorten the time of wet combustion of sea food rich in salt and cod liver oil by employing the "enclosed torch" devised at the same laboratory for the determination of iodine (which see). The ash is taken up in *nitric acid*, evaporated to small volume to remove chlorine, then, after addition of 5 to 20 ml. of *sulfuric acid*, further evaporated to fumes.

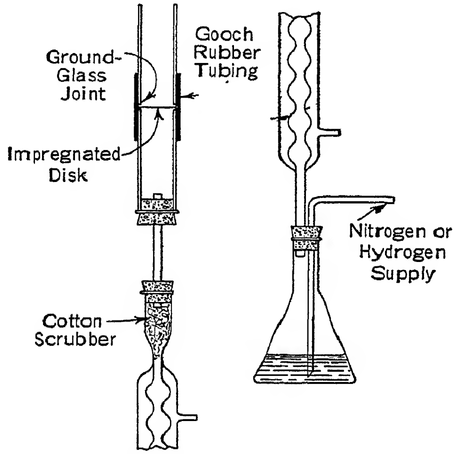
II. Lachele Mercuric Bromide Sensitized Disk Modification.[31] The modification, as used in the laboratories of the National Canners Association at San Francisco, is applicable for minute amounts in the presence of iron, tin, antimony, or reducible sulfur and phosphorus compounds. Mercuric bromide is used instead of the chloride.

Apparatus. *Disk Tube*, mounted over a reflux condenser which in turn is connected with scrubber, condenser, and an evolution flask as shown in Fig. 62. The cotton of the lower part of the scrubber is saturated with cuprous chloride solution.

Reagents. *Stannous Chloride Solution*, 40%, arsenic-free. Dissolve 40 g. of $SnCl_2 \cdot H_2O$ in HCl and make up to 100 ml. with the acid.

Mercuric Bromide Paper. Soak for 1 hour in a saturated ethanolic solution of $HgBr_2$ sheets of filter paper (similar to S & S No. 589 black ribbon), dry in an air blast, and cut into disks of the same diameter as the outer diameter of the diaphragm tube. Avoid contact with the hands and prepare fresh weekly.

Zinc, activated. Soak pieces 1 cm. long (about 5 g.) for 15 minutes in 1 + 3 HCl



Courtesy of Ind. Eng. Chem., Anal. Ed. 1934, **6**, *256*

FIG. 62. Lachele Arsenic Apparatus.

containing for each 100 ml. 2 ml. of the $SnCl_2$ solution, then wash thoroughly.

Ferrous Salt. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ or $FeSO_4 \cdot 7H_2O$ free from arsenic.

Cuprous Chloride Solution, 15%. Dissolve 15 g. of the salt in 100 ml. of 1 + 1 HCl.

Standard Arsenic Solution. Dissolve 1 g. of As_2O_3 in 25 ml. of 20% NaOH, saturate the solution with CO_2, and dilute to 1 liter with freshly boiled water (1 ml. = 1 mg. of As_2O_3). As needed, dilute 40 ml. of this stock solution to 1 liter, then 50 ml. of the latter to 1 liter (1 ml. = 0.002 mg. of As_2O_3).

PROCESS. *Arsine Evolution.* Place in a 1-liter Erlenmeyer flask an aliquot of the solution, obtained in the usual manner by digesting a weighed amount of the sample with *sulfuric* and *nitric acids*, and add water to a volume of 200 ml.; then add 2 to 3 g. of *solid ferrous salt*, 10 to 15 drops of *40% stannous chloride solution*, 50 ml. of *1 + 1 hydrochloric acid*, and finally 2 to 3 pieces of *activated zinc*. Connect quickly with the reflux condenser, start a flow of *nitrogen gas* through the assembly, and boil until all the arsine has been evolved (about 15 minutes).

Mercuric Bromide Development. Soak the disk in *cadmium iodide solution* until the red mercuric iodide, which first forms, has been dissolved, wash first with water, then with *ethanol*, and dry between blotters.

Color Comparison. Compare with *standards* representing amounts of arsenic trioxide in steps of 0.0025 mg., avoiding exposure to light.

MODIFICATION. The author also describes a modification applicable to undigested material and a special assembly for use in the presence of more than 30 mg. of antimony.

III. Cahill and Walters Mercuric Bromide Cotton Thread Modification.[32] As devised by two nuns of Regis College, Weston, Mass., cotton thread impregnated with mercuric bromide is used in a capillary tube.

APPARATUS. *Impregnating Device* (Fig. 63). The thread is drawn loosely over a glass rod bent to form a frame which fits into a cylinder.

REAGENT. *Standard Arsenious Oxide Solution.* Each 0.5 ml. contains 1 γ of the oxide.

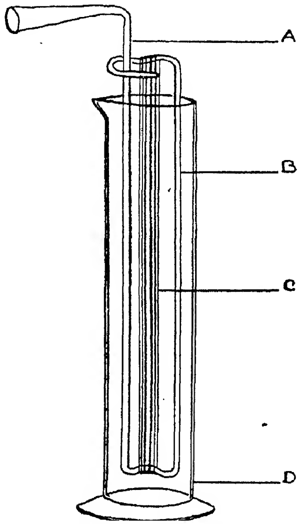
PROCESS. *Impregnation of Thread.* Soak No. 24 cotton thread with *4% mercuric bromide solution* for 15 minutes in the glass jar. Remove the frame, allow to dry, and cut the thread into lengths, handling each with forceps. Introduce a 0.1-mm. length into a 0.5-mm. bore capillary tube (No. 8 thread in a 1 ml. bore capillary tube was found to be less satisfactory).

Preparation of Scrubber. Soak glass beads overnight in *saturated lead acetate solution*,

pour on filter paper, and introduce into the scrubber.

Gas Evolution. See Sanger-Black Modification above.

Color Measurement. Measure the length of the stain and compare with that of a *standard* containing 1 γ of arsenious oxide per 0.5 ml.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1942, **14,** 91

FIG. 63. Cahill and Walters Device for Impregnating Threads for Arsenic Test.

Denigès Molybdate Reduction Colorimetric Method.[33] The renowned Denigès based a method, applicable to both phosphorus and arsenic, on the observation that both elements form with ammonium molybdate compounds which on reduction with stannous chloride, under certain circumstances, yield a blue color. Other reducing agents may be substituted for the stannous chloride with more or less success.

I. Truog and Meyer Modification.[34] After a study at the University of Wisconsin of the conditions favorable for the maximum color development, these authors prepared the two reagents as follows.

REAGENTS. *Acid Molybdate Reagent.* Dissolve 25 g. of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 200 ml. of water at 60° and filter. Dilute 280 ml. of arsenic- and phosphorus-free H_2SO_4 (about 36 *N*) to 800 ml. After cooling, add the molybdate solution to the acid slowly with shaking. Cool to room temperature and dilute with water to 1 liter. The solution is 10 *N* and contains 2.5% of ammonium molybdate.

Stannous Chloride Reagent. Dissolve 25 g. of $SnCl_2\cdot 2H_2O$ in 1 liter of 10% (by volume) HCl.

Filter, if necessary, and store in a bottle under a 5-mm. layer of white mineral oil with provision for delivering the undisturbed solution in drops.

PROCESS. The same process applies to both arsenates and phosphates. Arsenic may be separated as arsine and separately determined or both arsenic and phosphorus may be determined together, the arsenic removed, the phosphorus determined, and the arsenic obtained by difference.

If the organic matter is destroyed by ignition with magnesium nitrate, add *2.5% ammonium molybdate solution* and *10 N sulfuric acid* separately in the proper proportions; if the material is subjected to wet combustion, use the mixed reagents. In all cases adjust the amounts of the reagents in 100 ml., after the final dilution, to 4 ml. each of *2.5% molybdate solution* and *10 N acid* and 6 drops of *stannous chloride solution.* After adding each reagent mix thoroughly.

Color Comparison. Match the unknown with a *standard solution* within 10 minutes after adding the stannous chloride reagent.

II. Morris and Calvery Hydrazine Modification.[35] This modification (U. S. Food and Drug Administration) is a combination of (1) wet combustion with sulfuric, nitric, and perchloric acids, (2) a modified Marsh procedure, passing the gas through sand impreg-

nated with a little lead acetate before entering the combustion tube, and (3) spectrophotometric reading of the color formed with ammonium molybdate, hydrazine sulfate, and sulfuric acid similar to the procedure of Maechling and Flinn.[36] It differs from the Truog and Meyer modification chiefly in that hydrazine sulfate is substituted for stannous chloride as a reducing agent.

APPARATUS. A *Train* consisting of (1) a 15-ml. *gas reservoir* with a cock at the bottom, (2) a 50-ml. *Erlenmeyer flask* connected with the cock of the reservoir by a bent tube that passes through a two-hole stopper nearly to the bottom, (3) a *vertical condenser* bent at the upper part at right angles, (4) a *scrubber* containing sand impregnated with a few drops of 20% lead acetate solution, then dried, and (5) a *quartz combustion tube* (20 to 30 cm. of 5-mm. bore, 10 cm. of 1-mm. bore) heated in an electric furnace.

Spectrophotometer.

REAGENT. *Acid Molybdate Reagent.* Dissolve 1 g. of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 100 ml. of 5 N H_2SO_4.

PROCESS. *Digestion.* Weigh a suitable amount of the sample into a 1-liter round-bottom flask with three necks for standard taper connections. Join one neck with a downwardly arranged condenser and a receiver for trapping any escaping arsenic. Connect the other two with dropping funnels. Add 20 to 30 ml. of a mixture of *1 + 1 nitric and sulfuric acids* per 10 g. of sample, heat to foaming, and add dropwise *nitric acid* (usually 25 to 50 ml.) through one funnel during the concentration period. When the solution is complete, add dropwise but rather fast 4 to 6 ml. of *70% perchloric acid* through the other funnel, while continuing the addition of *nitric acid* through the first funnel, never allowing the color to turn brown, as best seen at the point of contact of the flame with the flask.

Perchloric acid is a dangerous explosive in the presence of large amounts of organic matter.

Continue the addition of *nitric acid* until the solution is water-clear, then heat 30 to 60 minutes longer for complete oxidation and removal of nitric acid. Finally dilute to a definite volume.

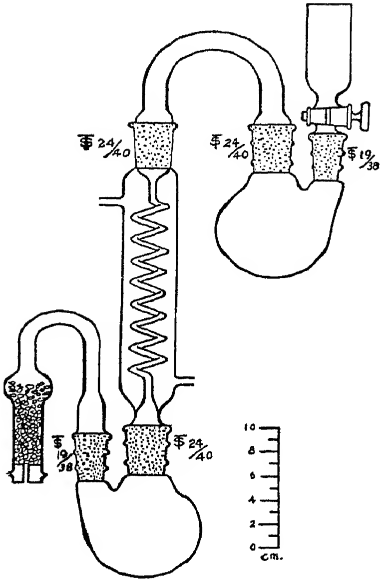
Arsine Evolution. Place 5 g. of *20-mesh zinc* and 1 ml. of *75% potassium iodide solution* in the Erlenmeyer flask of the train, introduce the two-hole stopper, immerse the flask to its neck in a water bath kept at 85°, and flush the system with *hydrogen* from a cylinder led into the reservoir of the train through a tube in a one-hole stopper. When thoroughly flushed, as shown by the usual test in an inverted test tube, heat the furnace and add to the flask an aliquot of 10 ml. or less of the unknown followed by *6 N hydrochloric acid* (usually 15 ml.) in portions sufficient to maintain a steady evolution of hydrogen for 45 minutes. Disconnect the quartz tube, cool under a jet of cold water, and dissolve the arsenic mirror in 0.2 ml. of *nitric acid,* rinsing into a 25-ml. Pyrex flask with about 5 ml. of water. Evaporate to dryness on the steam bath and dry in an oven at 120 to 125° for 1 hour.

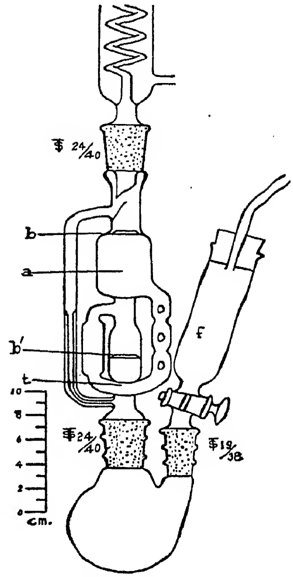
Color Formation. Dilute 10 ml. of the *acid molybdate reagent* to about 90 ml. with water and add 1 ml. of *hydrazine sulfate solution.* Make up to 100 ml. and mix. Add 10 ml. of the mixture to the unknown in the 25-ml. flask, attach a glass bulb, and heat for 10 minutes in boiling water.

Color Reading. Cool and read the color in the spectrophotometer at 6100 mμ in a 10-cm. tube of small bore.

CALCULATION. Obtain the weight of arsenic by comparison with a standard curve plotted from data obtained with known amounts of arsenic treated in the same manner as the unknown.

III. Chaney and Magnuson Hydrazine Distillation Modification.[37] By studies carried out at the College of Medical Evangelists and the University of Southern California, the method has been materially short-





Courtesy of Ind. Eng. Chem., Anal. Ed. 1940, **12,** *691 and 692*

FIG. 64. Chaney and Magnuson Digestion Assembly.

FIG. 65. Chaney and Magnuson Distillation Assembly.

ened and simplified by distillation as arsenic trichloride in a special assembly, thus dispensing with the deposition of the mirror in a combustion tube.

APPARATUS (Figs. 64 and 65). *Digestion and Distillation Assemblies.* (1) *Flasks,* round-bottom, two-neck, (2) *dripping funnel,* (3) *reflux condenser,* and (4) *special still* with still head *a,* sealed in lip below drip tip, baffle plate *b,* and trap *t* with indentations (K. D. Johnson, 1115 Arroyo Verde, South Pasadena, Calif.).

Heater, 350 watts (Cenco "hot cone"), variable transformer.

Photoelectric Colorimeter, with *tubes,* graduated at 25 ml.

REAGENTS. *Hydrazine Reagent.* Dissolve 5 g. of $H_2N \cdot NH_2 \cdot H_2SO_4$, 17 g. of KCl, and 4 g. of KBr in water and dilute to 100 ml.

Standard Arsenic Solutions. Dissolve 1.319 g. of reagent grade As_2O_3 in 15 ml. of 1.0 *N* NaOH. Dilute, add 15 ml. of 1.0 *N* HCl, and make up to 1 liter. This stock solution contains 1 mg. of arsenic per milliliter. Prepare standards containing 0.05 and 0.01 mg. per ml.

PROCESS. *Digestion* (Fig. 64). To 5 to 20 g. of tissue in a two-neck round-bottom flask, add 10 ml. of *nitric acid,* 5 ml. of *sulfuric acid,* and small pieces of *alundum.* Connect with the condenser by a U-tube and attach to the condenser a second flask with a soda lime tube. Heat on an electric heater (about 60 volts) and add *nitric acid* from time to time to prevent charring. When the yellow color indicates nearly complete digestion, add 0.5 ml. of *60% perchloric acid* through the dropping funnel. Cool and dilute with 10 to 15 ml. of water, also added from the dropping funnel. Boil vigorously at full voltage until

all the perchloric acid has been driven off and sulfuric fumes are evolved.

Distillation (Fig. 65). After cooling the digest, add 5 ml. of water, attach the dropping funnel (*f*) containing 2 ml. of *hydrazine reagent*, connect the still, and place on the hot 350-watt heater. When boiling begins and steam condenses in the trap, add 3 ml. of *0.3% potassium iodate solution* through the top of the still into the trap, attach the condenser, blow in the contents of the dropping funnel, and wash down with 2 ml. of water. Continue the distillation 4 to 6 minutes until iodine vapors condense in the condenser. Disconnect the still and pour the contents of the trap through the top into the 25-ml. graduated tube. Rinse the trap two or three times with 3 ml. of water to a final volume of 15 to 17 ml. The distillate should be faintly yellow, owing to free halogen. The arsenic in the distillate, as a result of oxidation, is in the pentavalent form.

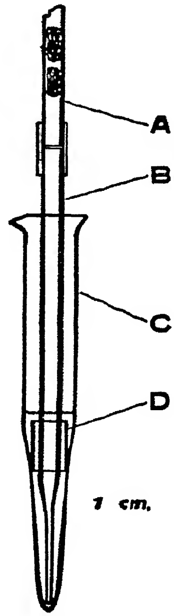
Color Formation. Place the graduated tube in a boiling water bath, add 2 ml. of *0.5% ammonium molybdate solution*, followed by 2 ml. of *0.15% hydrazine sulfate solution*. Continue the heating 10 minutes, cool, and dilute to 25 ml.

Color Reading. Using a 725 filter and a 1- or 2-cm. cell, read in a photoelectric colorimeter. Prepare in the usual manner the *standards*, containing 0.01 to 0.05 mg. of arsenic, adding to each 5 ml. of *1.0 N hydrochloric acid* and 0.3 ml. of *0.3% potassium iodate solution*.

IV. Sandell Mercuric-Permanganate Modification.[38] As practiced at the University of Minnesota, the arsine is absorbed in an acid solution of mercuric chloride containing permanganate and thus oxidized in one step to arsenate. Molybdenum blue is then formed by adding an excess of molybdate hydrazine reagent.

Apparatus (Fig. 66). A *Laboratory-Made Assembly* of a 50-ml. Erlenmeyer flask connected by a double-bent tube, containing two plugs of glass wool (*A*) impregnated with lead acetate, with a tube (*B*) narrowed at the end for delivering the arsine into an absorption vessel (*C*). A short piece of glass tube (*D*) with an inside diameter 1 mm. greater than the outside diameter of *B* breaks up the gas into bubbles.

Photoelectric Colorimeter.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1942, **14,** *82*

Fig. 66. Sandell Arsenic Absorption Apparatus.

Reagents. *Stannous Chloride Reagent.* Dissolve 40 g. of $SnCl_2 \cdot 2H_2O$ in 100 ml. of HCl.

Potassium Permanganate Solution, 0.03 *N*. Dissolve 0.1 g. of $KMnO_4$ in 100 ml. of water. Discard when a precipitate of MnO_2 forms.

Molybdate-Hydrazine Reagent. (*A*) Dissolve 1 g. of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 10 ml. of water and add 90 ml. of 6 *N* H_2SO_4. (*B*) Dissolve 0.15 g. of $H_2N \cdot NH_2 \cdot H_2SO_4$ in 100

ml. of water. Mix daily 10 ml. each of *A* and *B*, diluting to 100 ml. with water.

Standard Arsenic Solution, 0.1%. Dissolve 0.1320 g. of As_2O_3 in 2 or 3 ml. of 1.0 *N* NaOH, dilute with water, acidify with HCl, and make up to 100 ml. From this stock solution prepare a standard solution containing 0.01 mg. of arsenic per milliliter.

PROCESS. *Arsine Evolution.* Place in the 50-ml. Erlenmeyer flask a volume of the solution of the sample, conveniently 25 ml., containing not more than 15 γ of arsenic (20 γ of arsenious oxide). Add sufficient *hydrochloric acid* to make its total volume 5 ml., also 2 ml. of *15% potassium iodide solution* and 0.5 ml. of *stannous chloride reagent*. Allow to stand for 15 to 30 minutes at room temperature for reduction of the arsenic from the quinquivalent to the trivalent form, or heat to 80 to 90° and maintain at that temperature for 5 minutes, then cool to room temperature.

Measure 1 ml. of *mercuric chloride solution*, 0.2 ml. of *6 N sulfuric acid*, and 0.15 ml. of *0.03 N potassium permanganate solution* into the absorption vessel, mix with a thin glass rod, and connect the delivery tube with the double-bent tube and lower into the absorption vessel so that its tip nearly touches the surface of the solution, taking care that none of the absorption liquid enters the tube.

Quickly add 2 g. of *zinc* to the flask, insert the stopper, then lower the delivery tube so that it nearly touches the bottom of the vessel. Allow the gas to bubble through the solution for 25 to 30 minutes without heating; the solution should still contain an excess of the permanganate. Ignore a turbidity of hydrated manganese dioxide that forms when more than 10 ml. of arsenic is present.

Color Formation. Disconnect the delivery tube, leave it in the absorption vessel, add 5 ml. of *molybdate-hydrazine reagent*, mix, and heat for 15 minutes in a water bath at 95 to 100°. When cool, transfer the solution to a 10- or 25-ml. volumetric flask, make up to the mark with water, and filter through glass wool, rejecting the first portion of the filtrate.

Color Measurement. Determine the transmittancy in a photoelectric colorimeter, using a red filter showing the maximum transmission at about 700 mμ. In the comparison cell place the same amounts of reagents as in the unknown and treat in like manner, except that only 0.10 ml. of permanganate solution is added and the heating is for only 5 minutes.

Color Reading. Since the solution obeys Beer's law, plot a curve, using a single standard solution containing 0.001% of arsenic (as arsenious oxide) treated like the reagent solution. Introduce corrections for the blank.

Bettendorff Stannous Chloride Test,[39] Modified by King and Brown.[40] Work done at the Iowa State College demonstrated that when an excess of nearly *saturated stannous chloride solution* is added to a solution of an arsenical compound in a high concentration of *hydrochloric acid*, a brown coloration appears which changes, if the amount of arsenic is sufficient, to a black precipitate. Addition of *mercuric chloride* to a concentration of 0.00001 *M* before adding the stannous chloride hastens the appearance of the coloration and increases the sensitivity of the Bettendorff test ten- to a hundred-fold.

Taber Bromate Volumetric Method.[41] In 1934 this method was adopted by the Association as provisional for samples of convenient size for digestion that will yield 0.005 grain (0.324 mg.) of As_2O_3.

APPARATUS. *Distilling Assembly.* (1) a long-necked 800-ml. Kjeldahl flask, (2) a distilling tube (without condenser), bent at an angle of 70° and the long arm (40 to 50 cm.) drawn to an orifice of 3 mm., and (3) a 300-ml. Erlenmeyer flask. Boil the rubber stopper, connecting the flask with the short arm (10 cm.) of the distilling tube, successively for 15 minutes in 10% sodium hydroxide solution and hydrochloric acid.

Reagents. *Oxalate-Urea Reagent.* To a saturated solution of $(NH_4)_2C_2O_4 \cdot H_2O$, add 50 g. of urea per liter.

Hydrazine-Bromide Reagent. Dissolve 20 g. of $H_2N \cdot NH \cdot H_2SO_4$ and 20 g. of NaBr in 1 liter of 1 + 4 HCl.

Standard Bromate Solution. Dissolve 0.1823 g. of K_2BrO_3 in water and dilute to 1 liter. Standardize by titration at 90° against standard As_2O_3 solution in the presence of about 100 ml. of water and 25 ml. of HCl to simulate conditions under which samples will be treated (1 ml. = 1 ml. of As_2O_3 solution).

Standard Arsenious Oxide Solution. Dissolve 0.3241 g. of As_2O_3 in 25 ml. of 10% NaOH solution, acidify slightly with 1 + 6 H_2SO_4, and dilute to 1 liter.

Process. *Digestion.* To the sample containing 0.005 grain (0.324 mg.) or more of arsenious oxide in the 800-ml. Kjeldahl flask, add 35 to 50 ml. of *nitric acid,* then cautiously 20 or 25 ml. of *sulfuric acid,* and digest with addition of small amounts of *nitric acid* until the organic matter is destroyed and copious sulfur trioxide fumes are evolved. Cool slightly, add 50 ml. of water and 25 ml. of *oxalate-urea reagent* to aid in expelling oxides of nitrogen, and again evaporate to fumes. Dilute with 25 ml. of water, cool to room temperature, add 20 g. of *common salt* and 25 ml. of *hydrazine-bromide reagent,* and distil over a small flame into 100 ml. of water in the Erlenmeyer flask. Carefully adjust the flame so that the temperature of the distillate will rise to 90° in 9 to 11 minutes and adjust the heat so as to leave at least 55 ml. in the distilling flask.

Titration. To the distillate, containing arsenic trichloride carried over with the hydrochloric acid gas, add 3 drops of *methyl orange indicator* and titrate with *standard bromate solution* very slowly with agitation until a single drop destroys the final tinge of red color. Add 2 drops more of the *indicator;* this should produce a red color persisting at least 1 minute.

Blank. Correct for a blank determination on 5 g. of pure sucrose; this should not require more than 0.7 ml. of bromate solution. To test the sulfuric acid, bring 20 ml. to a boil, cool, dilute to 100 ml. with water, add a little *hydrochloric acid,* and titrate while hot.

Calculation. Use the formula: 1 ml. of standard bromate solution = 0.005 grain of As_2O_3.

Note. If the distillation proceeds too far or too much sulfuric acid is used in the digestion, sulfur dioxide is distilled and titrated as arsenic. If such contamination is suspected, dilute the titrated distillate to a definite volume and determine arsenic in an aliquot by the Gutseit method.

Zinzadze Molybdenum Blue Colorimetric Method.[42] Deemer and Schricker [43] applied the molybdenum blue reagent, as prepared by Zinzadze for the determination of phosphorus, to the determination of arsenic in food distillates. The following details are essentially as given by Zinzadze (New Jersey Experiment Station).

Apparatus. *Colorimeter.*

Reagents. See Zinzadze Molybdenum Blue Colorimetric Method for phosphorus, Part I, C8*a.*

Standard Arsenic Pentoxide Solution. Dissolve 0.02 g. of pure As_2O_5 in about 5 ml. of 2% $NaHCO_3$ solution and 100 ml. of water, add 6 ml. of 1 *N* H_2SO_4 and 3 drops of 0.1 *N* $KMnO_4$ solution, and dilute to 1 liter; 1 ml. = 0.02 mg. of As_2O_5. Prepare suitable dilutions in 50-ml. volumetric flasks.

Process. *Color Formation.* Pipet 0.5 to 30 ml. of the standard or unknown solution, corresponding to 0.005 to 0.3 mg. of arsenic pentoxide, into a 50-ml. volumetric flask with a mark at 40 ml. Add 5 drops of *α-dinitrophenol* and neutralize with *2% sodium bicarbonate solution* or *1.0 N sulfuric acid* to a faint yellow color, then add 5 ml. of *ten-fold diluted molybdenum blue reagent.* Make up to 40 ml., mix, heat on the steam bath for 30 minutes, let cool, and make up to 50 ml.

Color Measurement. Compare with the *standard solution* in a visual or electric colorimeter.

Zinzadze Molybdenum Trioxide Reduction Colorimetric Method.[44] REAGENTS. See Osmond-Zinzadze Molybdenum Trioxide Method for phosphorus, Part I, C8*a*.

PROCESS. *Color Formation.* Treat the standards and the unknown as in the method for phosphorus up to the addition of the molybdenum blue reagent, for which 5 ml. of the *molybdenum trioxide reagent* is substituted. Dilute to 40 ml., mix, then add rapidly 5 ml. of *stannous chloride solution* with shaking, and make up to 50 ml.

Color Reading. After 20 minutes, compare or read as in the foregoing method.

Klein and Vorhes Modification.[45] The procedure (U. S. Food and Drug Administration) is based in part on the two Zinzadze methods above and in part on the findings of Tarugi and Sorbini.[46]

APPARATUS. *Photometer.*

REAGENTS. *Zinzadze Reagent.* See method above.

Xanthate Reagent. To 10 parts by volume (ml.) of absolute ethanol in a centrifuge bottle, add 1 part by weight (g.) of pure NaOH pellets, warm to about 40°, and shake well for 15 minutes. Centrifuge and pour off the supernatant solution. Titrate with standard acid and adjust to 7% W/V NaOH by adding absolute ethanol. To 9 volumes of the adjusted solution, add slowly with cooling 1 volume of colorless CS_2, mix, and filter. The solution contains about 22% of sodium xanthate.

Extraction Reagent. Mix 50 ml. of the xanthate reagent with 1 liter of CCl_4. Prepare fresh each day.

Standard Arsenic Solution. Dissolve 1 g. of Bureau of Standards As_2O_3 in 25 ml. of 30% NaOH solution, neutralize with dilute H_2SO_4, and dilute to 1 liter. Dilute 50 ml. of this solution to 500 ml.; 1 ml. = 0.1 mg. of

PROCESS. *Solution.* Prepare a solution of a suitable weighed portion either (1) by wet combustion, dilution with 50 ml. of water, addition while still hot of 20 ml. of *10% potassium iodide solution,* and transfer to a 250-ml. separatory funnel with 50 ml. of water, or (2) by addition to an aliquot of a direct solution in a separatory funnel (containing not more than 0.8 mg. of As_2O_3 and not exceeding 200 ml. in volume) of one-fifth the volume each of *sulfuric acid* and while still hot *10% potassium iodide solution.*

In either case, allow to cool somewhat and oxidize the liberated iodine with *0.1 N sodium thiosulfate solution,* avoiding an excess of more than 0.5 to 1.0 ml.

Carbon Tetrachloride Extraction. Regardless of the volume, add 25 ml. of *extraction reagent,* stopper quickly, and shake vigorously for 1 to 2 minutes, then allow to stratify. Antimony is indicated by a brick red or yellow precipitate at the interface, and tin or copper by a deep yellow to orange coloration or precipitate in the lower layer.

Stannous Chloride Reduction. Draw off the lower layer into a previously dried 125-ml. separatory funnel containing 50 ml. of *hydrochloric acid* and 1 ml. of *40% stannous chloride solution,* rinsing with a little *carbon tetrachloride* without shaking so that any residual extract in the stem is displaced by the tetrachloride. Stopper and shake the second funnel for 2 minutes or until the lower layer assumes a very pale clear yellow color and the upper layer is either clear or contains only a slight amount of suspended matter, then draw off the layer through a plug of cotton 1/2 inch long, packed loosely into the stem of the funnel, into a third separatory funnel containing 50 ml. of *1 + 200 sulfuric acid.* Shake vigorously, draw off the lower layer through a plug of cotton into a 125-ml. Erlenmeyer flask, with a mark showing 60 ml., containing 10 ml. of water and 2 to 3 small glass beads.

Repeat the entire extraction with two ad-

ditional 15-ml. portions of the *extraction reagent*, added to the first funnel and carried through the process, then finally combine all three extracts.

Bromination. Evaporate the carbon tetrachloride on a hot plate and bring the aqueous solution to a vigorous boil for at least 1 minute with swirling. Rinse down the sides with a little water, add 20 ml. of *bromine water*, and heat on an asbestos board so as to reach gentle boiling in 2 to 5 minutes, then boil further until the color due to bromine is almost dispelled, and finally boil at full heat to remove the last traces.

Color Formation. Dilute to 60 ml., add from a pipet 10-ml. of *Zinzadze reagent* (tenfold dilution), bring to a boil, and boil gently for just 5.5 minutes. Cool, transfer to a 100-ml. volumetric flask, dilute to volume, and mix.

Color Measurement. Fill a 1-inch cell with the blue solution and take the reading in the neutral wedge photometer (see Clifford and Wichmann).[47]

Bromine Blank. To the remaining solution, add about 1 ml. of *bromine water*, warm until only a yellow color remains, take a photometer reading, and subtract from the direct photometer reading.

Calibration of Photometer. This is carried out with a series of 6 or 8 standards (0 to 0.8 mg. of As_2O_3) treated like the unknown, but omitting the hydrochloric acid-stannous chloride washing.

With a photometer equipped with a Wratten neutral gelatin wedge and a glass filter consisting of 4.5-mm. Corning dark pyrometer red No. 241, a linear relationship is obtained between photometer reading and arsenic content.

CALCULATION. To obtain the milligrams of As_2O_3 extracted (Y), solve the equation

$$Y = a + bX$$

by the method of least squares thus:

$$b = \frac{\Sigma XY - \Sigma XM_y}{\Sigma X^2 - \Sigma XM_x}$$

and

$$a = M_y - bM_x$$

in which Σ is "sum of" and M "mean of"; thus, ΣX is the sum of the X's; M_x is the mean of X; M_y is the mean of Y; ΣXY is the sum of the products of X and Y; ΣX^2 is the sum of the squares of X; and X is the photometer reading in millimeters (corrected for the bromine blank).

Exclude blanks (zero arsenic) from these calculations.

Cassil and Wichmann Arsine-Mercuric Chloride Iodometric Method.[48] The original method covers a range of 5 to 500 γ.

Cassil Rapid Modification.[49] The range of the original method is extended to 10 mg. of As_2O_3. Equations representing the reactions follow:

$$As_2O_3 + 6H_2 \longrightarrow 2AsH_3 + 3H_2O\ .$$

$$2AsH_3 + 12HgCl_2 + 3H_2O \longrightarrow \text{arsenides} \longrightarrow$$
$$12HgCl + As_2O_3 + 12HCl$$

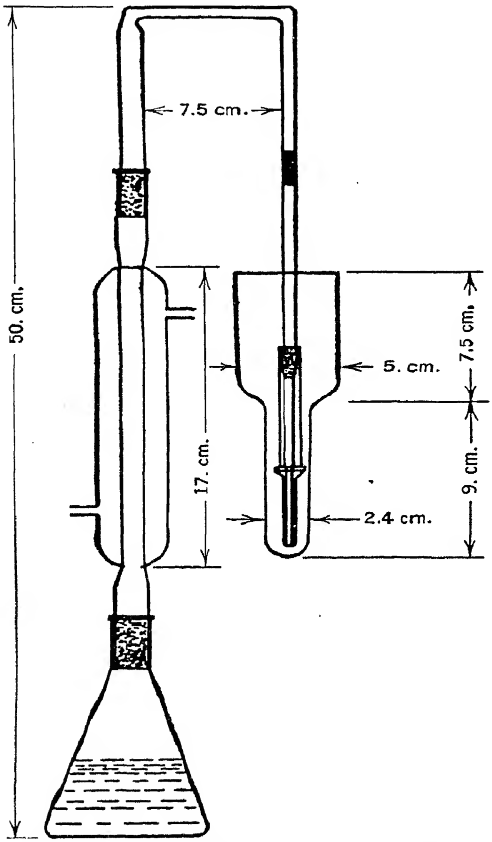
$$12HgCl + 24KI \rightarrow 6Hg^{\circ} + 6K_2HgI_4 + 12KCl$$

$$6Hg^{\circ} + As_2O_3 + 8I_2 + 12KI + 2H_2O \longrightarrow$$
$$+ As_2O_5 + 4HI$$

Although the mercury and arsenic are oxidized by the iodine, in effect this is the same as titrating arsine to arsenic oxide, 1 As being equivalent to 8 I.

APPARATUS. *Evolution, Absorption, and Titration Assembly* (Fig. 67). The parts are: (1) 125-ml. *Erlenmeyer flask* fitted with 24/40 standard taper ground-glass joint serving as the generator, (2) 18-cm. water-cooled *condenser* with 18/38 ground-glass joint on the upper end, (3) *adapter* fitted to the upper end of the condenser and connected at the other end by a 10/30 ground-glass joint to (4) the *delivery tube* with an end made of methyl methacrylate resin to prevent sticking of the mercury arsenide to the inside, provided with

(5) *baffle* to aid in stirring, and (6) *receiver* with a constricted end 24 mm. in diameter and 90 mm. long and upper end 50 mm. in outside diameter and 75 mm. long, the opening of the lower end being not over 2 mm. in diameter.



Courtesy of J. Assoc. Official Agr. Chem. 1941, **24**, 198

FIG. 67. Cassil Arsenic Assembly.

The adapter is filled with a loosely packed wad of dry glass wool, previously saturated with the lead acetate reagent, which is replaced from time to time as it becomes black in the lower half from lead sulfide.

A glass tube, with a 1-mm. opening, may be substituted for the methyl methacrylate resin delivery tube. It should be inserted into the absorbing liquid immediately after connecting to the condenser or when the flow of gas starts, thus preventing the absorbing liquid from backing up in the delivery tube.

REAGENTS. *Stannous Chloride Reagent.* Dissolve 40 g. of arsenic-free $SnCl_2 \cdot 2H_2O$ in 100 ml. of HCl.

Absorbing Solution. Dissolve 1.6 g. of $HgCl_2$ (reagent grade) in about 60 ml. of water, heating if necessary, cool, add 20 ml. of 2% aqueous U.S.P. gum arabic solution, and dilute to 200 ml.

Lead Acetate Reagent. Dissolve 10 g. of $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ in 80 ml. of water, make just acid to litmus paper with acetic acid, and dilute to 100 ml. with water.

Buffer Solution. Dissolve 10 g. of $Na_2HPO_4 \cdot 12H_2O$ in water and dilute to 100 ml.

Standard Iodine Solution, approximately 0.05 *N*. Dissolve 6.35 g. of pure iodine and 60 g. of KI in a small quantity of water, filter, and dilute to 1 liter. The excess of KI is used to form the K_2HgI_4 complex when added to the absorbing solution.

Standard Arsenic Solution. Dissolve exactly 2.5 g. of standard As_2O_3 in 25 ml. of 20% NaOH solution, saturate with CO_2 or neutralize with H_2SO_4, and dilute with water to 1 liter; 1 ml. = 2.5 mg. of As_2O_3.

Starch Indicator. Mix 1 g. of finely powdered soluble starch with cold water to a thin paste, add to 200 ml. of boiling water, and immediately remove the heat. The solution keeps indefinitely if stored with 1 ml. of metallic mercury.

PROCESS. *Standardization of Iodine Solution.* Add to the receiver 7 ml. of *iodine solution*, 15 ml. of water, and 10 ml. of *buffer solution*, then titrate to a colorless end-point with *standard arsenic solution*, using *starch indicator* when near the end-point. Then titrate in the receiver 20 ml. of *absorbing re-*

agent, 7 ml. of *iodine solution*, and 10 ml. of *buffer solution*. Agreement of the two titrations checks the purity of the reagents.

Calculate the titer of the iodine solution (T) as follows:

$$T = \frac{(A/A') \times A'}{I \times 4} = \frac{A}{I}$$

in which A is the milligrams of As_2O_3, A' is the milliliters of As_2O_3 solution, I is the milliliters of iodine solution, and 4 is the factor necessitated because in the method As^{---} is titrated to As^{+++++}, but in standardizing As^{+++} g As^{+++++}

Arsenic Isolation. Place in the generating flask an aliquot (not exceeding 50 ml.) equivalent to 0.50 to 10.00 mg. of As_2O_3, add *hydrochloric acid* to make the total quantity about 10 ml. and 5 ml. of *15% potassium iodide solution*. Dilute to 80 to 90 ml., then add about 1 ml. of *stannous chloride reagent*. Place 20 ml. of the *absorbing solution* in the receiver and connect to the train. Add 4 to 5 g. of *20-mesh granulated zinc* to the generating flask and immediately connect to the condenser, then heat nearly to boiling as rapidly as possible and remove the heat for the remainder of the 5-minute evolution period.

Titration. Disconnect the receiver and delivery tube, add through the latter an excess (at least 7 ml.) of the *standard iodine solution*, and stir until the solution clears. Add 10 ml. of *buffer solution* and titrate the excess of iodine to a colorless end-point with *standard arsenic solution*, using *starch indicator*.

Calculation. Obtain the milligrams of As_2O_3 (M) by the formula

$$M = [I - (A \times F)] - (A' \times T)$$

in which I is milliliters of iodine solution used in the actual determination, A is milliliters of As_2O_3 solution, F is the factor, A' is the milliliters of iodine solution used in the blank determination, and T is the titer.

Bambach Mercuric Chloride Hydroxylamine Polarographic Method.[50] The description which follows assumes some knowledge of polarographic technique. The steps in the procedure, as developed at the University of Cincinnati, are (1) isolation and concentration of the arsenic by the evolution of arsine and its absorption in mercuric chloride in a manner similar to that described by Cassil[51] and by Cassil and Wichmann,[52] (2) change of mercury arsenides to arsenious oxide by heating with an excess of mercuric chloride, (3) precipitation of mercury by reduction with hydroxylamine, and (4) polarographic determination.

Apparatus. *Leeds & Northrup Electro-Chemograph.*

Process. *Extraction.* Destroy the organic matter by wet combustion as described by Hubbard[53] and remove nitrogen oxides with *saturated ammonium oxalate solution* as in the Gutzeit method. Use the whole sample or an aliquot.

Isolation. Mix the solution with 10 to 15 ml. of *hydrochloric acid* in a 125-ml. Erlenmeyer flask, dilute with water to about 70 ml., then add 5 ml. of *potassium iodide solution*, 1 ml. of *stannous chloride solution*, and 10 to 12 g. of *stick zinc*. Fit to the flask of a Kjeldahl trap connected with a scrubber containing 100 ml. of *10% lead acetate solution*, and this in turn with a glass delivery tube that dips to the bottom of a 15-ml. graduated centrifuge tube containing 2 ml. of *1.6% mercuric chloride solution*. Heat the flask on a hot plate or on a water bath for about 30 minutes. Remove the centrifuge tube together with the delivery tube, heat in a water bath for 5 minutes, and remove the delivery tube, rinsing with hot water.

Concentrate the solution to about 2 ml., add 1 drop of *0.2% bromothymol blue solution* and 0.2 ml. of *40% hydroxylamine sulfate solution*, then while hot *ammonium hydroxide* in small drops until the mercury precipitates, at first in flocks, then, with more

alkali, in a finely divided black form. Avoid too great alkalinity as indicated by a blue color. The solution should be of a yellow-green color with a pH of 6. Heat until evolution of nitrogen ceases, cool, and note the volume.

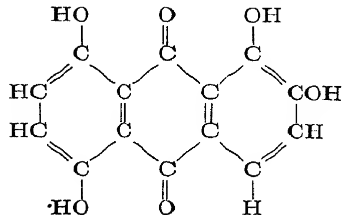
Polarographic Determination. Place 2 ml. of the clear supernatant liquid in the electrolysis cell, add 0.4 ml. of *9 N hydrochloric acid* and bubble *nitrogen* through the solution for no longer than 5 minutes, then polarize the mixture under *nitrogen* from 0 to −0.7 volt. If the quantity of arsenic is expected to be less than 20 γ, as judged from the appearance of the mercury arsenides in the precipitation tube during the previous step, use *3 N acid* instead of *9 N*. The half-wave of the arsenic step occurs at −0.35 volt, with the standard calomel electrode in *1.5 N hydrochloric acid* and at −0.5 volt in *0.5 N acid*. Measure the step height and compare with a calibration curve made by adding known amounts of *standard arsenious oxide solution* to 2-ml. portions of the *mercuric chloride solution* contained in centrifuge tubes treated as directed above at the beginning of the preceding paragraph. Make all determinations at the same temperature with the same capillary and drop rate. These precautions are essential since it is not possible to use an internal standard because of the complexity of the arsenic curve.

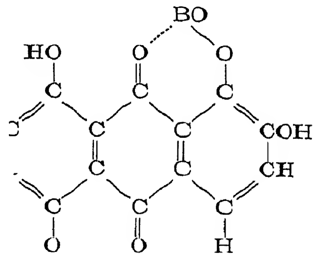
ERRORS. Below 10 mg., the error is 1 γ; above 10 ml., it is 3 to 5%.

BORON

The methods which follow are for the minute amounts naturally present in foods. Methods for larger amounts added as preservatives are described in Part I, C13.

Berger and Truog Quinalizarin Colorimetric Method.[54] Feigl and Krumholz [55] brought out that quinalizarin is suited for the detection of small amounts of boron; Berger and Truog (University of Wisconsin) devised a quantitative method, employing the same reagent, adapted for soils and foods. The reaction with boric acid results in the formation of a chelated compound as follows.





APPARATUS. *Color Comparison Tubes,* 100 mm. x 20 mm.

REAGENTS. *Sulfuric Acid,* 98.5%. Keep the strength between 98 and 99% by weight, as follows. Determine separately the strength, in terms of weight of SO_3 per gram, of H_2SO_4 (s) and fuming sulfuric acid (s') by titrating about 2 g. (w) of each acid with 1 N alkali and introducing the number of milliliters (v) required for the neutralization in equation (1). Mix as many grams of both acids (x = concentrated, $100 - x$ = fuming) as found by solving equation (2), 80.4 being the sulfur trioxide content of 98.5% H_2SO_4.

$$(1) \qquad s \text{ or } s' = \frac{v \times 0.04003}{w}$$

$$(2) \qquad sx + s'(100 - x) = 80.4$$

Before titrating the fuming acid, drop the stoppered weighing bottle (25 ml.) containing the acid into a larger stoppered weighing

bottle (100 ml.) containing 30 ml. of water, removing the stopper of the latter and stoppering in quick succession. After allowing to stand overnight or until fuming ceases, unstopper the two weighing bottles, place them in a liter beaker containing 300 to 400 ml. of water, and titrate.

Sulfuric Acid, about 0.36 *N*. Make up 5 ml. of H_2SO_4 to 500 ml.

Quinalizarin Reagent. Dissolve 0.01 g. of quinalizarin in 100 ml. of H_2SO_4, prepared by mixing 9 volumes of 98.5% H_2SO_4 with 1 volume of water, and store in a glass-stoppered bottle.

Standard Boric Acid Solution. Dissolve 2.8578 g. of boric acid in 1 liter of water. This solution contains 0.5 mg. of boron per milliliter. By diluting 20 ml. to 1 liter, and 100 ml. of the latter to 1 liter, solutions are obtained, containing 0.01 and 0.001 mg. of boron per milliliter respectively, to be further diluted in preparing comparison solutions.

PROCESS. *Incineration*. Burn in a platinum or porcelain dish 0.25 to 0.50 g. of the air-dry or oven-dried finely ground material at a low heat to a white or gray ash. Berger and Truog demonstrated that the same results are obtained with and without the addition of potassium carbonate prior to burning, even when there is a preponderance of acidic ash constituents. Cool, moisten with 5 ml. of *0.36 N sulfuric acid* accurately measured from a pipet, and triturate.

Color Formation. After settling, remove 1 ml. of the clear supernatant liquid to a comparison tube, add 9 ml. of *98.5% sulfuric acid*, stopper, and cool. Finally add 0.5 ml. of the *quinalizarin reagent*, stopper, mix by gentle whirling, and allow to stand at least 15 minutes.

Color Comparison. Compare with a set of *standard solutions* containing 0.0002 to 0.0040 mg. of boron prepared by accurately measuring into comparison tubes portions of stock solutions of *boric acid*, diluting each to 1 ml. with water, and treating with reagents in the same manner as in the actual analysis.

Cassal and Gerrans Oxalic Acid-Curcumin Colorimetric Method.[56] Little had been written on this method, since it was announced in 1903, until Naftel (Alabama Agricultural Experiment Station) combined its essential features with certain steps of the classical Gooch Method,[57] although the latter was not designed to determine traces in plants and oils.

Naftel Modification.[58] APPARATUS. *Colorimeter*.

REAGENTS. *Oxalic-Hydrochloric Acid Reagent*. Mix daily 20 ml. of HCl and 80 ml. of a saturated solution of $H_2C_2O_4 \cdot 2H_2O$.

Curcumin Solution, 0.10% in ethanol, or *Turmeric Tincture*, 1%, prepared daily by shaking the ground rhizome for 4 to 6 hours and filtering.

PROCESS. *Oxalic-Hydrochloric Acid Treatment*. Place an aliquot of a plant ash or soil extract, containing 0.5 to 8 γ of boron, in a porcelain evaporating dish, add 5 ml. or more of *0.10 N calcium hydroxide suspension*, and evaporate to dryness at full heat on a water bath. Cool to room temperature and cool also the water bath to 55° ± 3°.

Color Formation. To the cooled residue, add 1 ml. of the *oxalic-hydrochloric acid reagent* and 2 ml. of *curcumin solution* or *turmeric tincture*. Rotate the dish until mixed and evaporate to dryness at 55°. Continue the heating for 30 minutes at that temperature, then allow to cool. Extract the residue with *ethanol* and transfer to a filter or a 15-ml. centrifuge tube. Filter and wash thoroughly with *ethanol* or centrifuge for 10 minutes at 1500 r.p.m. and wash. Dilute the liquid phase to 25 ml.

Color Comparison. Make the comparison in a 20-ml. colorimeter cell against a *standard boric acid solution* treated in like manner or read the concentration on a standard curve plotted with 0 to 70 scale readings as ordinates and 0 to 0.40 γ/g. of boron as abscissas.

Wilcox-McCormick Electrometric Titration Method.[59] The method, as applied by Wilcox to irrigation waters, has been adapted by McCormick, of the Montana Experiment Station, to plant material.

APPARATUS. *pH Apparatus*, with silver chloride and platinum electrodes.

PROCESS. Reduce 5 g. of the finely ground, air-dry sample to ash by heating for 16 hours at 350 to 400°. Wash the ash into a 550-ml. beaker of boron-free glass with water, followed by 2 ml. of *nitric acid*, then add about 4 ml. of the *acid* to acid reaction. Heat 10 minutes on a steam plate, cool, and dilute to 100 ml.

Barium Precipitation. Add 12.5 ml. of *1.0 N barium chloride solution* and make slightly alkaline to *phenolphthalein* with *13 N sodium hydroxide solution.* Add *nitric acid* to faint acid reaction, then add *saturated barium hydroxide solution* to a faint, enduring pink color. Heat to boiling and filter immediately through a retentive paper such as Munktell No. 3, and wash the precipitate 7 times with hot water. To the filtrate add 18.75 ml. of *2 N sodium chloride solution,* bringing the chloride ion concentration to 0.2 equivalent per liter. Dilute to 250 ml. and make slightly acid to *bromothymol blue* with *nitric acid*, adding 1 drop in excess, and boil for 3 minutes to expel carbon dioxide. Cool and nearly neutralize to the *bromothymol blue* with *carbonate-free 0.5 N sodium hydroxide solution.*

Electrometric Titration. Add sufficient *quinhydrone* (0.2 g.) to saturate the solution. Introduce the silver chloride and platinum electrodes which are connected to the galvanometer through a tap key. Add *carbonate-free sodium hydroxide solution* until a null point is indicated on the glavanometer. This is the initial point of the titration and is at pH 7.6. Add 10 g. of *mannitol* and titrate with *standard carbonate-free 0.0231 N sodium hydroxide solution* (standardized against boric acid) until the null point is again reached.

NOTE. All glass ware, as well as reagents, must be boron-free.

BROMINE

See Part II, A2, D2, and F1.

COBALT

Although cobalt occurs in minute amount in foods, there is no convincing evidence that it is essential to health.

Ahmad and McCollum Nitroso R Salt Colorimetric Method.[60] APPARATUS. *Colorimeter.*

REAGENT. *Nitroso R Salt Solution,* 0.2%. Dissolve 0.2 g. of 1-nitroso-1-hydroxy-naphthalene-3,6-disulfonic acid in 100 ml. of water.

PROCESS. *Incineration and Solution.* Burn to a white or gray ash an amount of the sample representing 20 g., dissolve in *hydrochloric acid,* and evaporate the solution to near dryness. Take up in 20 ml. of hot water and 0.5 ml. of *hydrochloric acid* (sp.gr. 1.12), boil, and filter. Boil the filtrate 5 to 10 minutes with 5 drops of *50% nitric acid* and cool.

Color Formation. Add 3 g. of *sodium acetate* and 1 ml. of *0.2% nitroso R salt solution,* warm to 70°, and allow to stand 5 minutes to develop the color.

Color Comparison. Make faintly alkaline to *phenolphthalein* with *10% potassium hydroxide solution,* then distinctly acid with a few drops of *hydrochloric acid.* Heat to boiling, add dropwise 2 ml. of redistilled *50% nitric acid,* boil 2 minutes, dilute to 25 ml., and compare with a *standard cobalt solution* treated in like manner.

EXAMPLES. White corn 0.068, yellow corn 0.06 to 0.07, wheat 0.063 to 0.15, soy beans 0.236, peanuts 0.57, pecans 0.25, beans 0.18 to 0.475, smooth field pea 0.16 to 0.24, red cow pea 0.274, whole milk powder 0.06, skim milk powder 0.046 to 0.07, and pancreas 0.18 to 0.24 γ/g., dry basis.

Copper

See also Part II, F3, and G1.

Aside from its natural occurrence in all animal and vegetable foods, copper may be present in fruits and vegetables, together with arsenic, as a highly objectionable constituent of certain spray residues and in manufactured foods as salts derived from utensils and containers. Formerly it was used for greening vegetables and pickles, but that practice has been discontinued in the United States, owing to federal prohibition.

Cereals and oil seeds are reported to contain 2 to 20 γ/g. and wheat germ and bran as high as 16 γ/g.; root and tuber vegetables show an even wider range, the maximum being over 50 γ/g., and leaf vegetables may contain as high as 40 γ/g., fresh basis, but the high figure may be due to spray residue. Milk contains 0.05 to 0.6, lean meat 1 to 5, and the edible portion of fish up to 4 γ/g., fresh basis. Drained oysters range from 6 to 600 γ/g., fresh basis, according to locality; near a copper refinery nearly 1500 γ/g.

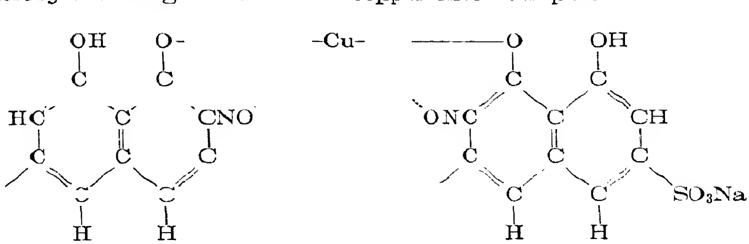
Early Methods. Serger [61] and other earlier workers used a colorimetric method based on the formation of deep blue cuprammonium when the *copper solution* is mixed with an excess of *ammonium hydroxide.* Others used *potassium ferrocyanide* as the reagent, the precipitate formed being of a red-brown color. Maquenne and Demoussy [62] and Guérithault [63] increased the delicacy of the test by employing 2 drops of *1% zinc sulfate solution* and 1 drop of fresh *potassium ferrocyanide solution,* thereby obtaining a blue color.

The more recent methods depend on color reactions with organic reagents, notably *pyridine* in conjunction with *potassium thiocyanate,* and azo compounds, such as *potassium ethyl xanthate, ammonium nitrosochromotropate* (chromotropic method), and *sodium diethyldithiocarbamate* (carbamate method).

After destruction of the organic matter, it was found necessary to separate the copper from interfering substances, by precipitation by hydrogen sulfide or electrolysis, before titration or color comparison as described below.

Scott and Derby Xanthate Colorimetric Method.[64] Pipet into each of two colorimetric comparison tubes 10 ml. of *0.1% potassium ethyl xanthate solution.* To one tube add an aliquot of the solution (prepared according to the chromotropic method below) and to the other tube add from a buret an approximately corresponding amount of *standard copper sulfate solution.* Make both up to the mark, shake, and compare in a colorimeter or match the color in the solution of the sample with one of a series of Nessler tubes containing a suitable range of amounts of copper.

Brenner Chromotropic Volumetric Method. Brenner [65] showed that micro titration with *chromotropic acid* in ammoniacal solution is applicable in the determination of nickel and copper, as well as of cobalt which was his special problem, also that various azo dyes may serve in a similar manner in the determination of iron, silver, zinc, cadmium, strontium, calcium, magnesium, and barium. He gives the following formula for copper chromotropate:



I. Cherbuliez and Ansbacher Modification.[66] The Brenner method was adapted for use in food analysis at the University of Geneva.

II. Ansbacher, Remington, and Culp Modification.[67] A further adaptation of the chromotropic method was developed at the Medical School of the University of South Carolina.

REAGENTS. *Standard Copper Sulfate Solution.* Accurately weigh out into a 500-ml. volumetric flask 4.91 g. of $CuSO_4 \cdot 5H_2O$ of highest purity, freshly recrystallized and thoroughly dried with filter paper, dissolve in water, and make up to the mark. Pipet 5 ml. of this stock solution into a 250-ml. volumetric flask and make up to the mark. One milliliter of the last solution theoretically contains 0.05 mg. of copper; if, however, doubt exists as to the purity and water content of the copper sulfate, determine the copper electrolytically in 25 ml. of the first solution which should contain 0.0625 g. of copper.

Standard Chromotropic Solution. Dissolve 0.37 g. of the sodium salt of nitrosochromotropic acid (1,8-dihydroxy-2-nitroso-3,6-naphthalenedisulfonic acid) in a small amount of water, add 1 ml. of 2 N Na_2CO_3, 0.5 to 0.6 ml. of 2 N $NaNO_2$, and a slight excess of dilute acetic acid. Filter after 24 hours, add Na_2CO_3 to weakly alkaline reaction, dilute to 100 ml. with water, mix, remove 34 ml. to a 1-liter volumetric flask, dilute, add 50 ml. of ethanol, and make up to the mark. In standardizing, mix in a test tube 1 ml. of the standard copper sulfate solution with 3 ml. of ammonium hydroxide and titrate with the standard chromotropic solution as in an actual analysis.

PROCESS. *Solution.* A wet combustion process for relatively small amounts of the sample and an incineration process for larger amounts are in substance as follows.

A. Wet combustion is carried out on 10 to 20 g. of milk, meat, and fresh vegetables and fruits and on 1 to 2 g. of dry fruits, vegetables, and seeds. To the substance in a Kjeldahl flask, moistened with water if dry, add 15 ml. of *sulfuric acid.* Cover with a funnel and boil until the mass blackens and sulfur trioxide fumes appear. When cool, add 5 ml. of *20% perchloric acid* and 2 ml. of *fuming nitric acid.* Heat gently until nitrogen tetroxide fumes cease to escape, then heat until white fumes appear. If not colorless, after cooling repeat the treatment with *perchloric acid* and *nitric acid* and heating. Remove the solution to a small Erlenmeyer flask and dilute to an acid content of at most 15% by volume.

B. Incineration is employed when 20 to 40 times the amount used in wet combustion is available. Char a weighed portion in a silica dish, then heat for 30 minutes in a muffle furnace at dull redness or below 400°. Cool, add *fuming nitric acid,* and evaporate to dryness on a hot plate. If little organic matter remains after cooling, add more *fuming nitric acid* and again evaporate to dryness. If, however, considerable organic matter remains unburned, repeat the heating in the muffle furnace and the treatment with *fuming nitric acid.* Heat the ash in the silica dish with 10 ml. of *sulfuric acid,* cool, dilute, remove to an Erlenmeyer flask, and dilute to an acid content of 15% by volume.

Sulfide Precipitation. Heat the solution prepared by one of the foregoing methods to boiling, add a few drops of *nitric acid,* and pass a current of *hydrogen sulfide* through a double-bored stopper into the liquid until it is cold. Allow to settle, filter through a small crucible with a porous bottom (Central Scientific Co., No. 3810, fine), wash with water acidified with *acetic acid* and saturated with *hydrogen sulfide* until all iron is removed, keeping the crucible covered with a glass plate to exclude air. Place the crucible on a glass triangle over a small crystallizing dish resting on a glass water bath, add *fuming nitric acid* to the crucible, and wash with water after the acid has completely passed

through. Evaporate the solution to dryness on the water bath and heat on a dry hot plate until fumes cease to appear, taking care that the copper nitrate is not decomposed. Dissolve the residue in water, dilute to 25 or 50 ml. in a volumetric flask, and remove an aliquot of 1 to 5 ml. to a test tube for the titration.

Titration. To the aliquot, add 3 drops of *ammonium hydroxide* and titrate in daylight with the *standard chromotropic solution.*

CALCULATION. Obtain the copper content in terms of milligrams per kilo or liter. Comparison with solutions showing the color before (permanganate red) and after (brown) the end-point is reached aids in the titration.

Sheets, Pearson, and Gieger [68] secured the sharpest end-point when a 5-ml. aliquot contains 0.003 to 0.015 mg. of copper.

EXAMPLES. Lettuce 11.2, apples 9.8 to 12.4, dried milk 1.2 to 2.0, egg yolk 7.4, and oysters 64.1 γ/g.

Biazzo Pyridine-Thiocyanate Colorimetric Method.[69] The method is based on a reaction discovered by Spacu.[70] Two other color reactions are described by the same author.[71] One with *toluidine* (2 drops of 2% ethanolic solution) and *potassium thiocyanate solution* (few drops) yields a blue precipitate, the other with *potassium iodide solution* (2 ml.) and *benzidine* (3 drops of 1% solution in ethanol) yields a dark blue precipitate.

The Biazzo method is briefly as follows. Ash 5 g. of the sample, dissolve the ash in *50% hydrochloric acid*, evaporate on the water bath, take up in water and transfer to a separatory funnel, then dilute to about 20 ml. Add *sodium hydroxide solution* until alkaline to *phenolphthalein*, acidify with *acetic acid*, then add a few drops of *concentrated potassium thiocyanate solution* and a little *pyridine*. Extract the mixture with 5 ml. of *chloroform* and compare the color of the chloroform solution with a *standard copper sulfate solution* treated in like manner.

NOTES. Schönheimer and Oshima [72] describe a method similar to that of Biazzo.

Kleinmann and Klinke [73] use, instead of 5 ml. of chloroform, 1 ml. of *bromobenzene* (b.p. 154 to 155°) in conjunction with 25 drops of *acetic acid*, 1 or 2 ml. of *pyridine*, and 3 ml. of *10% ammonium thiocyanate solution.*

Elvehjem and Lindow Modification.[74] Details as reported from the University of Wisconsin follow.

PROCESS. *Wet or Dry Combustion and Separation.* As under the Chromotropic Method above.

Color Comparison. To one of two 25-ml. separatory funnels, add an aliquot of the neutral solution prepared as above; to the other add from a pipet an amount of *standard copper sulfate solution* suitable for color comparison, as judged from the appearance of the hydrogen sulfide precipitate. Adjust both to the same volume and add 10 ml. of water, 1 ml. of *glacial acetic acid*, and 10 drops of *pyridine*, then 1 ml. of *10% potassium thiocyanate solution* and exactly 5 ml. of *chloroform.* Shake both thoroughly, allow to settle, and draw off the bromobenzene into slender color-comparison tubes.

CALCULATION. Obtain the copper content from the relative color intensity of the columns.

Clarke and Jones Dioxime Colorimetric Method.[75] The method consists briefly in adding to the slightly acid copper solution (which must be free from chloride) 1 g. of *ammonium persulfate*, 1 ml. of *saturated dimethylglyoxime solution*, 0.5 ml. of *0.5% silver nitrate*, and 2 ml. of *10% pyridine* in water. The permanganate pink color is compared with that produced with a solution containing a known amount of copper treated in like manner.

Hurd and Chambers,[76] of Wisconsin University, have carefully studied the method and report that it is not suitable for general use because of the many factors contributing

to the color variation. If used, it is suggested that a series of secondary permanganate standards be established.

Callan and Henderson Carbamate Colorimetric Method.[77] The method employs a new reagent, *sodium diethyldithiocarbamate*, which gives a brown precipitate with copper. The reaction is stated to be more sensitive than that with potassium thiocyanate or potassium ferrocyanide. The procedure in substance consists in adding to the ammoniacal solution containing the copper 10 ml. of *0.1% solution of the carbamate* and dilution to 100 ml.

NOTES. McFarlane[78] extracts the color with *amyl alcohol*, Thatcher[79] with *isoamyl alcohol*, Williams[80] with *chloroform*, and Haddock and Evers[81] with *carbon tetrachloride*.

Conn, Johnson, Trebler, and Karpenko Modification. Details of the method as applied to milk are given in Part II, G1.

Walker Thiosulfate Iodometric Method (Minimum 1 Milligram of Copper).[82] Thiosulfate is used first in saturated solution for the precipitation of the copper and later as a standard solution in the usual iodometric titration.

REAGENTS. *Sodium Thiosulfate Solution*, concentrated. Prepare a hot saturated solution.

Standard Sodium Thiosulfate Solution, 0.01 *N*. Dissolve 24.82 g. of $Na_2S_2O_3 \cdot 5H_2O$ in 1 liter of CO_2-free water to make an approximately 0.1 *N* solution. After standing (preferably 2 weeks), dilute to 0.005 or 0.01 *N* with CO_2-free water. Standardize daily by titrating against 20 ml. of standard copper solution made alkaline with NH_4OH as in the process below under *Titration;* 1 ml. of 0.01 *N* sodium thiosulfate = 0.6357 mg. of copper.

Standard Copper Solution. Dissolve 318 mg. of pure metallic copper in HNO_3, evaporate to dryness on the steam bath, dissolve the cuprous nitrate thus formed in water and a few drops of acetic acid, again evaporate to dryness, and dilute to 1 liter in a volumetric flask.

PROCESS. *Thiosulfate Precipitation.* Dissolve the ashed charge in *hydrochloric acid*, or prepare a solution by wet digestion, and neutralize with *ammonium hydroxide.* Add 5 ml. of *sulfuric acid*, dilute to 200 ml., and boil for 1 minute, then add cautiously 10 ml. of hot *saturated sodium thiosulfate solution.* Continue the boiling for 5 minutes with the addition of a few milliliters of *1% ammonium sulfate solution*, if the amount of copper is small, to aid coagulation. Filter and wash six times with hot water. If desired, reserve the filtrate for determination of zinc. Ignite the paper with precipitate in a muffle furnace at 500°. Treat the residue with 1 ml. of *2 + 5 nitric acid* and evaporate on the steam bath. Add 5 ml. of water and again evaporate to dryness, then take up in 20 ml. of water, add an excess of *ammonium hydroxide*, and heat on the steam-bath until the copper has dissolved. Transfer to a 100-ml. Erlenmeyer flask.

Titration. Boil gently to drive off the excess of ammonium hydroxide, acidify to *litmus paper* with *1 + 1 acetic acid*, add 1 ml. in excess, boil for 1 minute, and cool to room temperature. Add 2 g. of *potassium iodide* dissolved in water to make a final solution of 50 ml. and titrate immediately with *0.01 or 0.005 N sodium thiosulfate solution*, depending on the intensity of the blue color, until near the end-point. Finally add 2 ml. of *starch solution* and continue the titration to the disappearance of the blue color. Compare the color with that of the titrated standard.

Electrolytic Precipitation of Copper. For this separation, a solution is prepared by heating the ash (see Chromotropic Method above) with a small amount of *1 + 5 nitric acid*, evaporating nearly to dryness, and adding *3% by volume sulfuric acid* to the desired bulk, depending on the amount of ash and the apparatus. Employ a roll of platinum gauze or a filter cone for an electrode and a

current of 2.5 to 3.0 volts and 0.25 ampere. Heat nearly to boiling and run the current for 10 or 15 minutes or until the liquid fails to respond to the ferrocyanide test. Continue the current while washing the electrode. Dissolve off the copper with a small amount of *nitric acid*, evaporate to dryness, and take up the residue in hot water.

If the amount warrants, weigh the electrode with its deposit of copper on a delicate balance, dissolve the copper in *nitric acid*, weigh again, and calculate the copper in terms of gammas per gram. In case the quantity is minute, evaporate the copper solution to dryness in a porcelain dish, dissolve the residue in *sulfuric acid*, dilute, and determine the copper volumetrically or colorimetrically by one of the foregoing methods.

Fluorine

See also Part II, F1.

Although it has long been known that fluorine occurs in teeth, there appears to be conclusive evidence that a certain amount is essential for sound dentation and normal nutrition. On the other hand, it has been shown that drinking water and foods with abnormal amounts are toxic.

Willard and Winter Zirconium Alizarin Sulfonate-Thorium Nitrate Volumetric Method.[83] De Boer and Basart [84] describe a volumetric method for the analysis of fluorspar (CaF_2) involving refluxing the unknown and the blank with zirconium oxychloride solution, addition of sodium alizarin sulfonate as indicator, titration with potassium fluoride, and calculation of the fluorine by difference. Willard and Winter (University of Michigan and Michigan Agricultural Experiment Station) use zirconium alizarin sulfonate as indicator and standard thorium nitrate solution for the titration.

Reagents. *Zirconium-Alizarin Indicator.* (*A*) Dissolve 1 g. of $Zr(NO_3)_4 \cdot 5H_2O$ in 250 ml. of water. (*B*) Dissolve 1 g. of sodium alizarin sulfonate in 100 ml. of ethanol. Filter and add 150 ml. of ethanol to the filtrate. As needed, mix three parts of *A* and two parts of *B*. The mixed reagent should be violet-red.

Standard Thorium Nitrate Solution, 0.01 *N*. Standardize against standard NaF solution.

Standard Lithium Fluoride Solution, 0.01 *N*, or *Standard Sodium Fluoride Solution*, prepared from the specially purified salt.

Process. *Distillation.* To a suitable amount of the sample in a small distillation flask, add a few glass beads or pieces of porous plate, 5 ml. of *60% perchloric acid*, and water to 110° boiling point. Close with a rubber stopper carrying a thermometer, a capillary tube connected with a dropping funnel, and an exit tube, then distil into an open container, holding the temperature of the solution in the flask at 135° by adding water through the dropping funnel from time to time.

Color Formation. When all the fluorine has passed over (50 to 75 ml.), add to the distillate 6 drops of *zirconium-alizarin indicator* and *sodium hydroxide solution* dropwise until the color of the indicator appears, then add an equal volume of *ethanol* and *1 + 50 hydrochloric acid* until the color just disappears. To make certain that the solution is slightly acid, add *alkali* and *acid* alternately to a suitable end-point.

Titration. Carry out the titration with standard *0.01 N thorium nitrate solution* over a white surface in a good light to the faint but permanent reappearance of the color. The reaction is slow at the end-point.

Correction. Titrate 6 drops of zirconium-alizarin indicator with *0.01 N fluoride solution* to the disappearance of the color, thus obtaining the number of milliliters of the standard solution that combines with the indicator, and correct the number of milliliters of standard solution used in the titration of the unknown accordingly.

I. Armstrong Alizarin Sulfonate Modification.[85] Armstrong (University of Minnesota) substitutes 3 drops of 0.05% aqueous sodium alizarin sulfonate solution for 6 drops of zirconium alizarin indicator and titrates in an aqueous (not ethanolic) solution.

A buffer is used because of its convenience, not as claimed by Hoskins and Ferris (see below) who first introduced this feature, because it is essential for accuracy, since by careful adjustment of the acidity with hydrochloric acid before titration satisfactory results are obtained.

Chloride is removed by silver perchlorate when the amount is sufficient to interfere with the end-point.

APPARATUS. *Distillation Assembly.*

Cylindrical Vials, about 4.5 x 1.4 cm.

REAGENTS. *Sodium Perchlorate.* Neutralize 60% $HClO_4$ with NaOH solution and evaporate to a boiling point of 140°. Chill the solution, collect the crystals, and dry at 110°.

Silver Perchlorate Solution. Prepare in the dark room from 40.3 g. of 60% $HClO_4$ and a slight excess of freshly prepared Ag_2O. Filter and dilute to 250 ml.; 5 ml. = 1 g. of the salt. Store in a dark bottle.

Buffer. Neutralize to phenolphthalein 50 ml. of 4 *M* monochloroacetic acid with NaOH solution, add 50 ml. of 4 *M* monochloroacetic acid, and dilute to 200 ml.

Standard Sodium Fluoride Solution, 1 mg. of fluorine per milliliter. From this stock solution prepare solutions containing 1, 2, 5, and 10 γ/ml.

PROCESS. A. SODIUM CHLORIDE LESS THAN 0.018 GRAM IN 10 MILLILITERS OF DISTILLATE. *Distillation.* Charge the 50-ml. distilling flask with a suitable portion of the sample, 3 glass beads, 0.1 g. of *acid-washed ignited sand,* 10 ml. of water, and 5 ml. of *60% perchloric acid,* also, if the charge contains 5 γ or less of fluorine, 6 g. of *sodium perchlorate.* Heat to 140° and collect 150 ml. of distillate. Maintain the distillate at just alkalinity during the operation. Evaporate the distillate, while still alkaline, to small volume, cool, and make just acid with *hydrochloric acid.* If 5 γ or more of fluorine are present dilute in a 10-ml. volumetric flask to the mark; if 5 γ or less, evaporate and adjust to exactly 5 ml.

Titration. Pipet 1-ml. aliquots into the cylindrical vials, add to each a rod and one drop of *0.05% aqueous sodium alizarin sulfonate solution.* Add split drops of *dilute (about 0.03 N) hydrochloric acid* until a light orange color appears, then add 1 drop of *buffer solution* and titrate with *0.0004 N thorium nitrate solution* delivered from a micro buret.

CALCULATION. Use the formula: 1 ml. of the 0.0004 *N* thorium nitrate = 7.3 γ of fluorine.

Standardize the thorium nitrate solution concurrently by the titration, as above directed, of standard fluoride solutions containing 1, 2, 5, and 10 γ of fluorine. The end-point is the first appearance of the pink color which matches that of the reference solution. Use the color obtained in the titration of 5 γ of fluorine in 1 ml. for reference in all titrations of unknown and standards.

Determine the titration blank by substituting the titration of 1 and 10 γ of fluorine in the 2-point equation for a straight line

$$\frac{y - y_1}{x - x_1} = \frac{y_1 - y_2}{x_1 - x_2}$$

and calculate the *y*-intercept or titration blank. From the milliliters obtained in the titration of 1, 5, and 10 γ of fluorine, calculate the mean equivalence of the thorium nitrate solution.

B. SODIUM CHLORIDE OVER 0.018 GRAM IN 10 MILLILITERS OF DISTILLATE. *First Distillation.* If soluble, dissolve the sample in 150 ml. of water and precipitate the chlorine as directed below. Perform the first distillation as described under A, but testing with *phenolphthalein paper* in maintaining the al-

kalinity of the distillate with *1.0 N sodium hydroxide solution*. Since the greater part of the hydrogen chloride is evolved during the initial boiling at 140° for 5 minutes, use *0.1 N sodium hydroxide solution* during the last half of the distillation. Make the distillate acid with *dilute perchloric acid*.

Chloride Precipitation. Add *silver perchlorate solution* in subdued light until the silver chloride coagulates, continuing the addition in 0.1-ml. portions until a drop added to the filter paper impregnated with *5% potassium chromate* produces a red color. Make the solution alkaline to *phenolphthalein paper* and coagulate the precipitate by heating to boiling, then cool and filter. Evaporate the filtrate to about 10 ml., filter to remove any silver oxide formed during the concentration, then evaporate the filtrate to about 5 ml. and transfer to the distilling flask.

Second Distillation. Add to the solution 4 to 6 g. of *sodium perchlorate* and perform the second distillation as above, maintaining the alkaline reaction of the distillate. Evaporate the distillate to small volume, acidify, and dilute to the desired volume. Shake for 3 minutes with 0.25 g. of *activated charcoal* per 10 ml. of solution, and filter through a dry paper.

Titration. Make the titration on a 1-ml. aliquot and calculation as directed under A.

Notes. Rowley and Churchill,[86] of the Aluminum Company of America, corroborate Armstrong's findings that titration in an aqueous solution is more satisfactory than in a 48% ethanolic solution, especially for the larger amounts of fluorine.

The tentative methods of the A.O.A.C. for the determination of fluorides in insecticides and fungicides [87] and in potable water [88] are adaptations of the Armstrong modification. An illustration shows a well-arranged assembly for steam distillation, consisting of (1) a steam evolution flask, with a pressure tube extended to the bottom, and (2) a Claissen distillation flask with a double-bored stopper carrying the steam entrance tube extended to the bottom and a thermometer, connected through the tubulature with a diagonally arranged condenser, both flasks being heated by Bunsen burners. No method designed for spray residues has been adopted.

II. Hoskins and Ferris Modification.[89] After an intensive study at the University of California of the Armstrong modification, it was concluded that the most favorable procedure includes the use of the indicator at a concentration of 4×10^{-5} per cent, in a total volume of 50 ml., titration to match a blank at a very light pink shade, and regulation of the *p*H of both the blank and sample at 3.5. The desired *p*H is secured by the use of a buffer system of *sodium hydroxide* and *chloroacetic acid* at a ratio of 0.5 and a total concentration of 0.02 *M*. The dissociation constant of the acid in 50% commercial ethanol is given as 2.8×10^{-4} *sodium alizarin sulfonate* in ethanol; in water it acts as an indicator over a range of *p*H 4.8 to 7.2 and 3.7 to 5.2 respectively.

An accuracy of about 99% was secured with 57 to 760 γ of fluorine in a 50-ml. solution and 6 to 90 γ in a 5-ml. solution.

III. Eberz, Lamb, and Lachele Modification.[90] Devised by workers at the Research Laboratory of the National Canners Association at San Francisco to determine fluorine spray residue on tomatoes, the essential features of the original method are combined with the sodium alizarin sulfonate solution of Armstrong and the chloroacetic acid buffer of Hoskins and Ferris. Also improvements in ashing, distillation, and end-point matching are introduced.

Reagents. *Standard Sodium Fluoride Solution*. Dissolve 2.2105 g. of NaFl in water and dilute to 1 liter. Dilute 10 ml. of the stock solution to 1 liter; 1 ml. = 0.01 mg.

Standard Thorium Nitrate Solution. Dissolve 0.4579 g. of $Th(NO_3)_4 \cdot 12H_2O$ in water and dilute to 1 liter.

Chloroacetic Buffer (Hoskins and Ferris),

0.24 *M*. (*A*) Dissolve 22.7 g. of monochloroacetic acid to give 100 ml. of solution. (*B*) Neutralize 50 ml. of *A* with 6 *N* NaOH solution. Mix *A* and *B* and dilute to 1 liter.

Silver Perchlorate Solution (Armstrong). Adjust the concentration so that 1 ml. is equivalent to 0.02 g. of NaCl.

PROCESS. *Incineration and Solution.* Incorporate 100 g. of finely ground tomatoes with 1 g. of *magnesium oxide of low fluorine content*, evaporate on the water bath overnight without stirring, then bake for 2 to 3 hours at 135° in an oven and finally incinerate for 15 minutes at 500°, covering the dish during the first 2 minutes with a sheet iron plate. Cool, macerate in 50 ml. of water, evaporate, and dry for 30 minutes at 135°, then incinerate as before.

Chloride Precipitation. Immediately on cooling, take up the ash in 50 ml. of hot water, add *silver perchlorate solution* dropwise until a brownish or yellowish color persists on stirring. Evaporate on the water bath, transfer to the distilling flask, first with a spatula, then with two 5-ml. portions of water, followed by 5 ml. of *6 N perchloric acid.*

Distillation. Add 24 ml. of purified *perchloric acid* and distil at 135 to 138° in the Willard and Winter apparatus. Insert into the water tube a capillary tube of such diameter as to allow a flow of 4 ml. per minute under a 35-cm. water head with the stopcock completely open, thus permitting adjustment by raising or lowering the separatory funnel. Neutralize the distillate with *0.1 N sodium hydroxide solution* to give a faint pink color with 1 drop of *phenolphthalein* and concentrate to 12.5 ml. or less, then transfer to a 50-ml. beaker.

Titration. Add 15 ml. of *ethanol,* 0.1 ($\pm$0.01) ml. of *0.05% sodium alizarin sulfonate solution*, and water to make 27.5 ml. Adjust the color with *0.05 N sodium hydroxide solution* and *0.5 N hydrochloric acid* until it is a pure yellow without excess of acid, then add 2.5 ml. of *0.24 M chloroacetic buffer* and titrate with *standard thorium nitrate solution* from a 5-ml. micro buret. The final volume should not be disturbed more than 1 ml. by the volume of the 0.5 *N* hydrochloric acid or the sodium hydroxide. Adjust the end-point to match that of a blank to which exactly 0.05 ml. of *standard thorium nitrate solution* has been added. Make the final matching in 50-ml. Nessler tubes on a white background illuminated by light from the blue sky, not direct sunlight. Since the colors are not stable, a carefully adjusted mixture of *cobalt nitrate* and *potassium chromate* is suitable for matching.

Armstrong Acetylacetone Colorimetric Method.[91] The fading action of fluorine on the color of ferric acetylacetone is made the basis of a method, developed at the University of Minnesota, in which the influence of the acidity and certain impurities are eliminated by determining the fading due to fluorine in an aliquot of the solution, followed by a measurement of the fading due to an equal aliquot containing added fluorine.

APPARATUS. *Bausch & Lomb Duboscq Colorimeter*, with No. 3610 light filter in the eyepiece.

REAGENTS. *Ferric Chloride Solution.* 0.03%. Protect from light and discard in 2 or 3 hours; 1 ml. = 0.03 mg. of iron.

Acetylacetone Solution, 0.5%. Prepare from freshly distilled acetylacetone.

PROCESS. *Adjustment of Acidity.* If the solution of the unknown contains carbonates, add a few drops of *phenolphthalein indicator*, heat to boiling, add to the boiling solution *0.1 N hydrochloric or nitric acid* dropwise until no pink color appears on further boiling, then immediately add *0.1 N sodium hydroxide solution* to a slight alkaline reaction and allow to cool in a stoppered flask. Whether or not this adjustment is made, make the solution of the unknown faintly acid, then add 1 drop of *1 + 100 acid.*

Color Formation. Place in two 25-ml. volumetric flasks 1 ml. each of *0.03% ferric*

chloride solution and *0.5% acetylacetone solution.* Add to one flask an aliquot of the unknown, containing not over 0.25 mg. of fluorine, and dilute the contents of both flasks to volume.

Color Comparison. Place the solution containing the unknown in the right-hand cup of the colorimeter and the solution containing no fluorine in the left-hand cup, set at 20 mm. Take 20 readings by increasing the depth in the right-hand cup and averaging the results. Repeat the procedure with the same size aliquot of the unknown, adding 1 ml. (0.1 mg. fluorine) of the *standard fluorine solution* to the flask containing the unknown. Again take 20 readings and average the results.

CALCULATION. Obtain the milligrams of fluorine (F) in the total solution by the formula

$$F = \frac{0.1D(X - 20)}{Y - X}$$

is the reading of the unknown, Y is the reading of the unknown plus 0.1 mg. of added fluorine, and D is the ratio of the total volume of the solution to the volume of the aliquots.

Kolthoff and Stansby Purpurin–Zirconium Oxychloride Volumetric Method.[92] The method was developed at the University of Minnesota for 0.5 to 15 mg. of fluorine.

REAGENTS. *Standard Acid Zirconium Oxychloride Solution.* Dissolve 0.8 g. of $ZrOCl_2 \cdot 8H_2O$ in 1 liter of 10 N HCl.

Cobalt-Dichromate Reagent. Dissolve 19.6 g. of $Co(NO_3)_2 \cdot 6H_2O$ and 0.132 g. of $K_2Cr_2O_7$ in water and dilute to 1 liter.

PROCESS. *Solution.* Introduce into a 100-ml. oil-sample bottle a quantity of the solution of the fluorine, separated from the sample by the Willard and Winter method (above) or as silicon tetrafluoride,[93] containing 0.5 to 15 mg. of fluorine. If solid, add 2 ml. of water; if liquid, adjust 2 ml. or a larger volume to 10 N with *hydrochloric acid,* and add 2 ml. of water. Add 5 ml. of *10 N hydrochloric acid* from a buret, then 2 ml. of *0.03% purpurin indicator in ethanol* from a pipet.

Titration. Measure 40 ml. of *cobalt-dichromate reagent* into an oil-bottle for a color standard. To the solution of the unknown, add *standard acid zirconium solution* from a buret slowly with shaking until the color (1 to 2 minutes) begins to approach that of the color standard, then add more *10 N hydrochloric acid* to a volume of a little below 40 ml. Finally complete the titration and adjust to exactly 40 ml. with *10 N hydrochloric acid.*

CALCULATION. From the number of milligrams of zirconium used in the titration, subtract 1 mg. to correct for the blank, then find the corresponding amount of fluorine in the following table.

FLUORINE CORRESPONDING TO ZIRCONIUM USED

Zirconium Used	Fluorine Present	Zirconium Used	Fluorine Present
mg.*	mg.	mg.*	mg.
1	0.40	15	6.63
2	0.75	16	7.15
3	1.20	17	7.73
4	1.60	18	8.26
5	2.00	19	8.90
6	2.47	20	9.50
7	2.90	21	10.10
8	3.25	22	10.72
9	3.70	23	11.40
10	4.15	24	12.08
11	4.65	25	12.90
12	5.10	26	13.70
13	5.60	27	14.53
14	6.12	28	15.40

* Minus 1 mg.

Kolthoff and Stansby Purpurin-Zirconium Oxychloride Colorimetric Method.[92] This method is for 0.01 to 0.05 mg. of fluorine.

REAGENTS. *Zirconium-Purpurin Reagent.* Add slowly with shaking a solution of 9 mg. of purpurin in 30 ml. of ethanol to a solution of 0.16 g. of zirconium oxychloride in 6 *N* hydrochloric acid, then add to the mixture 620 ml. of hydrochloric acid and dilute to 1 liter with water.

Standard Fluoride Solution, containing 2 mg. of fluorine per 100 ml. of 8 *N* HCl.

PROCESS. Measure 10 ml. of *zirconium-purpurin reagent* into each of two test tubes of uniform diameter. To one tube add the unknown made up to 2 ml. and 6 *N* with respect to hydrochloric acid; to the other tube add 2 ml. of *6 N hydrochloric acid.* Add 2.4 ml. of the *standard fluoride solution* from a micro buret to the second tube; this gives an orange color. Then add the same *fluoride solution* to the other test tube and enough *8 N hydrochloric acid* so that the colors of the two match for the same volume.

IODINE

The discovery of the relation of iodine in water and food to goiter and cretinism led to the study of the content of this element in animal and vegetable foods. At first qualitative tests were deemed sufficient, but it soon became apparent that it required little more labor to make the tests quantitative, after the iodine had been separated by tedious processes of combustion and extraction.

Colorimetric Methods. The only color reaction that has seemed practicable is that involving liberation of free iodine and observation of the violet color it imparts to chloroform, carbon tetrachloride, or carbon disulfide. Since color comparison is practically the same in all the colorimetric methods, originality is confined to details of combustion and extraction where loss may be total loss and the iodine in the blank may exceed that in the food.

Winterstein Open Combustion Colorimetric Method.[94] This semi-quantitative method (Zürich Technical Institute) was the precursor of the von Fellenberg method.

PROCESS. *Combustion and Extraction.* Mix 10 to 50 g. of the material with 8 times its bulk of *40% sodium hydroxide solution*, burn carefully to ash, take up in water, and nearly neutralize with *dilute sulfuric acid.* Filter, moisten the residue with *sodium hydroxide solution*, add a few pieces of *solid sodium hydroxide* and a little *nitric acid*, and burn. Take up in hot water, nearly neutralize with *sulfuric acid*, and add to the preceding filtrate. Evaporate the united solutions to small bulk, cool, acidify with *sulfuric acid*, neutralize accurately with *sodium hydroxide solution*, avoiding an acid reaction, evaporate to dryness, and heat at 100°. Boil the residue with *ethanol*, filter, and repeat the treatment, washing with *ethanol*. Evaporate the united ethanolic residues to dryness and heat gently.

Color Comparison. Dissolve the residue in 2 ml. of water, filter, wash with 1 ml. of water, shake with a measured small amount of *chloroform*, and determine the iodine colorimetrically.

EXAMPLES. By this procedure, Winterstein recovered 4 mg. of iodine added to 1 kilo of iodine-free spinach. He found traces in potatoes, but none in most cereals and vegetables, and none in milk and cheese, although later authors, working with greater precision, have reported definite amounts in all of these.

von Fellenberg Open Combustion Colorimetric Method.[95] The original paper of nearly eighty pages deals exhaustively with the occurrence of iodine in water, foods, and animal organs and describes in great detail, but none too clearly, the processes of incineration, extraction, and color comparison of the chloroform extract. The salient features of the process follow.

REAGENT. *Nitroso-Sulfuric Acid.* Add 0.05 g. of KNO_2 to 3 *N* H_2SO_4 and make up to 10 ml. with the acid.

PROCESS. *Combustion and Extraction.* Mix in an iron dish 1 to 3 kilos of the material and 7 g. of *calcium hydroxide* with enough water to make a thin paste, then with 3 ml. of *concentrated potassium carbonate solution* for each 100 g. of material; burn at the lowest possible heat. Autoclave hard seeds with water for 30 minutes at 2 to 3 atmospheres before burning. Cover the glowing portion of the material with the unburned portion to condense the iodide fumes. Add 2 to 3 ml. of *concentrated potassium carbonate solution* and repeat the burning. Extract the ash by boiling several times with a considerable amount of water, combine the extracts, and evaporate in a glass dish to near dryness. Extract the residue with several portions of *ethanol*, evaporate to small volume, nearly neutralize to *phenolphthalein* with *1 + 1 hydrochloric acid* to check foaming and prevent the silica in water solution from passing into the ethanol.

Color Comparison. Transfer to a special separatory funnel that fits in a cup of a centrifuge, add to the solution from a 0.1-ml. micro buret 0.02 ml. of *chloroform* and a drop of *nitroso-sulfuric acid*, turn end to end 80 times, and whirl. Transfer the chloroform extract to a special narrow tube and compare the color with that of a *standard solution*.

Allott, Dauphinee, and Hurtley Modification.[96] In this process useless details have been eliminated and a simple and ingenious device is employed for color comparison of the iodine. Harvey[97] prefers this modification, employing iodine-free potassium hydroxide solution prepared as described below. Although the method was designed, at St. Bartholomew's Hospital, London, for blood, it is equally well suited for various animal foods.

APPARATUS. The special stoppered tube has a mark for measuring 1 ml. The constriction at the bottom end is for the carbon disulfide solution of the liberated iodine.

REAGENT. *Potassium Hydroxide Solution, 10 N*, iodine-free. Harvey's method of preparation is as follows. Dissolve 125 g. of KOH to a saturated solution, add 0.25 g. of $H_2N \cdot NH_2 \cdot H_2SO_4$, boil for 30 minutes, cool, and shake in a separatory funnel with an equal volume of iodine-free acetone, which has been redistilled with KOH. Repeat the extraction 9 times, rejecting the acetone extract, then boil to expel dissolved acetone, cool, and dilute.

Carbon Disulfide. Treat with about 3% of pure iodine, let stand overnight, remove the iodine by shaking with NaOH, wash with water, dry over $CaCl_2$, and distil.

PROCESS. *Incineration, Extraction, and Solution.* Dry 10 ml. of blood or 10 g. of meat, etc., with 1.5 ml. of *10 N potassium hydroxide solution* in a nickel dish at 150° and burn at 500° in a muffle furnace. Extract 4 times, each with 4 ml. of water, filtering through three G4 Jena glass filters. Saturate the combined filtrates with *carbon dioxide* and evaporate to dryness in a platinum or nickel dish. Extract the residue 4 times with 3 ml. of *ethanol*, the second time with *absolute*, the other three with *95%*, decant carefully, and filter through a Jena glass filter. Evaporate the combined filtrates in platinum on a water bath.

Iodine Liberation. Heat the residue in a muffle furnace at 500° for 2 minutes, extract repeatedly with small portions of water to a total of 1 ml. collected in the special tube. Add a drop of *nitrosulfonic acid* to liberate the iodine and shake out with 0.05 ml. of *purified carbon disulfide*.

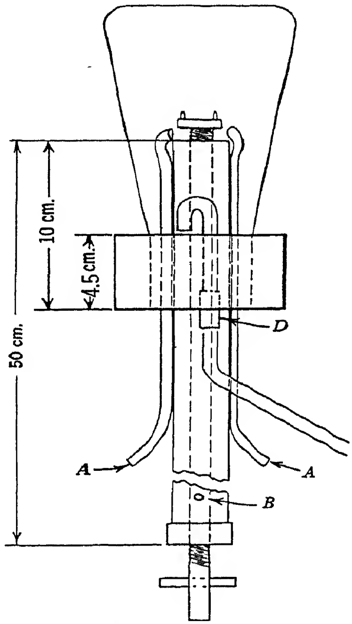
Color Comparison. Treat one or more *standard potassium iodide solutions* in the same manner and compare in the usual way.

Blanks must be made on all reagents.

McClendon-Remington Closed Combustion Colorimetric Method. McClendon[98] (University of Minnesota) devised a method obviating the losses of iodine sustained in the open combustion of certain materials,

that has been perfected with the collaboration of Remington, von Kolnitz, Rufe, and Culp.[99]

Von Kolnitz and Remington Simplification of the Karns Technique.[100] The special feature is the modification of the ignition flask apparatus devised by Karns.[101]



Courtesy of Ind. Eng. Chem., Anal. Ed. 1933, **5**, 39

FIG. 68. Von Kolnitz and Remington Iodine Apparatus.

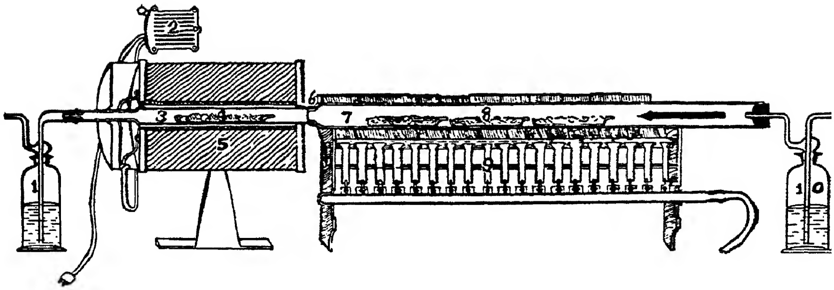
APPARATUS (Fig. 68). The essential parts are the wide-mouth Erlenmeyer flask inverted in a water-seal cup through which passes a cylinder, with an air vent 3 mm. in diameter (*B*), within which is the feed device that advances and rotates the candle of the material being analyzed, two brass oxygen feed tubes, 5 mm. outside diameter (*A*), and a glass outlet tube, 7 mm. outside diameter (*C*), connected with two Milligan absorbers. *D* is a rubber-tubing gasket for outlet tubes and *E* is a hole, 9 mm. in diameter, for the outlet tube. All the brass parts are coated with nitrocellulose lacquer.

PROCESS. *Combustion.* Pack the dried animal or vegetable material in a Visking sausage casing (2.35 cm. in diameter), thus forming a cartridge that moves freely as it is advanced by the threaded tube. Connect the outlet tube with two Friedrich wash bottles, the first containing 2 *sodium hydroxide pellets* dissolved in water and the second bottle only one *pellet*. Ordinarily the second serves merely to collect the spray containing only traces of iodine. For the combustion of dried milk or any ordinary products, employ 2 jets delivering 1 to 1.5 liters of *oxygen* per minute. For the combustion in exceptional cases, add two other jets.

Extraction. After the combustion, rinse the flask with water in the cup and combine with solutions in the Friedrich wash bottles. Test to insure alkalinity, evaporate to small volume, centrifuge to remove insoluble matter, and evaporate to dryness. Add an additional *sodium hydroxide pellet*, heat just to fusion, moving the dish over the flame. Extract thoroughly with *ethanol* added in several portions or dissolve in a small amount of water directly; this is satisfactory in many cases.

Color Comparison. Add a pinch of *sodium azide* to decompose nitrites, followed by a little *sodium sulfite* to reduce iodate, and then *phosphoric acid* until acid. Boil to expel sulfur dioxide and transfer to a separatory funnel. Add a minute crystal of *sodium nitrite* to liberate iodine, and 1 ml. of *carbon tetrachloride*. Shake thoroughly, separate the tetrachloride, and compare with a *standard solution* treated in the same manner.

McHargue, Young, and Calfee Closed Combustion Colorimetric Method.[102] In this process, developed at the Kentucky Agricultural Experiment Station, the quartz combustion tube is used with valid precautions to avoid loss.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1934, **6**, *319*

FIG. 69. McHargue, Young, and Calfee Iodine Assembly.

APPARATUS. *Assembly*, as shown in Fig. 69.

PROCESS. *Combustion.* Thoroughly mix in a porcelain dish 50 g. of the finely ground air-dry material with 10 g. each of finely pulverized *calcium oxide* and *copper oxide*. Distribute in three alundum boats Fig. 69 (8) and place in the silica combustion tube (7) which is connected at the right through a rubber stopper with the wash bottle (10) containing *10% potassium hydroxide solution* and at the left by an asbestos cement seal with a silica catalyst tube (3). Connect the catalyst tube containing platinized asbestos arranged for heating in an electric furnace with two wash bottles (only one shown in the figure) containing *5% potassium carbonate solution*. Attach the end bottle to a suction pump.

Switch on the current to the electric furnace and, when tube 3 attains a red heat, draw air through the system and light the first burner at the left of the gas furnace. When burning begins, gradually light one burner after another in proper sequence so as to keep a slow steady burning like a lighted cigar. Unburned vapors from the sample are completely burned in passing over the catalyst and iodine vapors are carried into the wash bottles and absorbed. When the combustion is complete, turn off all heat and cool while continuing the air current. Carefully remove the boats from the tube.

Extraction. Digest and leach the ash with hot distilled water, filter, combine the filtrate with *5% potassium carbonate solution* in the absorption bottles, and evaporate to dryness. Add sufficient water just to dissolve the ash, transfer to a separatory funnel, add enough *ethanol* to form two immiscible layers, and shake vigorously for 10 minutes. Run off the aqueous solution into another separatory funnel and repeat the process three times. Combine all ethanolic extracts and evaporate cautiously to dryness.

Color Comparison. Dissolve the residue in a few drops of water, filter into a small separatory funnel, and make slightly acid with *sulfuric acid*. Add 3 ml. of *saturated sulfurous acid solution*, stopper, and shake vigorously for 1 minute, thus reducing iodate to iodide. Measure accurately into the separatory funnel 1 ml. of *carbon disulfide* and 2 ml. of *10% sodium nitrite solution*, stopper, shake vigorously for 1 minute, and allow to settle. If the carbon disulfide has a slight pink color, all the iodine has been absorbed; if it has a pronounced pink color, run it into a centrifuge tube and repeat the shaking of the aqueous liquid with 1 ml. of *carbon disulfide*, repeating until the color is faint pink. Combine the carbon disulfide solutions, clear by centrifuging, and compare with a *standard solution* prepared in the same manner.

Volumetric Methods. The general processes of combustion and extraction given above under Colorimetric Methods are also applicable, preliminary to titration with sodium thiosulfate solution, to other natural foods provided the quantity of iodine present, as in certain animal and vegetable sea foods, is considerably more than faint traces. For example, in the edible portion of oysters and some other sea foods, as high as 7 γ/g., dry basis, of iodine has been reported. Special initial treatment is required for mixtures consisting largely or entirely of inorganic matter.

Kendall Iodate Volumetric Method.[103] In this Mayo Clinic method and the earlier Hunter method [104] advantage is taken of the following reaction, thereby increasing the delicacy of the titration six-fold over that without the addition of the iodine:

$$HIO_3 + 5HI \longrightarrow 3H_2O + 3I_2$$

In later methods, closed tube combustion is substituted for ignition in a nickel crucible and the iodine is extracted with ethanol.

PROCESS. *Combustion.* Evaporate to a sirup in a nickel crucible on a hot plate 2 or more grams of the sample, 5 ml. of *30% sodium hydroxide solution*, and 10 to 15 g. of *solid sodium hydroxide* in pieces. Place the crucible in a larger crucible containing a 0.5-cm. layer of *sand* and heat at dull redness. Rotate the crucible while cooling and adding 5- to 10-mg. portions of *potassium nitrate* until no gas is evolved. Pour the melt into the crucible cover, cool, and transfer both to a 600- to 800-ml. beaker, adding 125 to 150 ml. of water, and a little *talc*.

Extraction. Dissolve the melt by heating, transfer to a 500-ml. Erlenmeyer flask, and add 1 ml. of *10% sodium bisulfite solution* and a few drops of *methyl orange*. After cooling, add *85% phosphoric acid* in slight excess, then *bromine water* until the solution is distinctly yellow. Dilute to 250 to 300 ml., boil vigorously for 8 to 10 minutes, add 5 to 10 drops of *5% sodium salicylate solution*, and cool.

Titration. Add 5 ml. of *10% potassium iodide solution*, also 3 to 4 ml. of *85% phosphoric acid* if necessary, and titrate with *standard 0.005 N sodium thiosulfate solution*, using *starch solution* as indicator.

CALCULATION. In calculating the results, divide the iodine corresponding to the thiosulfate used by 6.

NOTE. Bohn [105] substitutes *Congo red* for methyl orange, adding the acid to a distinct blue color. He found the Krauss palladous chloride method [106] less reliable.

I. Knapheide and Lamb Modification.[107] Halvorson and others [108] demonstrated that this modification (Moorman Mfg. Co.) is suited for small amounts of iodine in medicinal cattle feeds, provided special attention is paid to the initial steps.

REAGENT. *Reduced Phosphoric Acid,* 20% solution. Dilute 85% acid with 4 volumes of water and boil for some time with aluminum strips as described by Kendall.[109]

PROCESS. *Combustion.* Fuse together in a 100-ml. nickel crucible 20 g. of *sodium hydroxide* and 10 g. of *potassium nitrate*, cool, place 10 g. of the sample evenly over the melt, and moisten well with 5 ml. of *saturated sodium hydroxide solution* and 10 ml. of *80% ethanol*. Cautiously heat on a hot plate for 30 minutes to remove the ethanol, continue the heating at the highest temperature attainable on the plate for 90 to 120 minutes, and finally with constant watching fuse in a suitable furnace that heats sides and bottom, taking care to avoid loss during the violent reaction of the charcoal with potassium nitrate. When the fusion reaches the quiet stage, add small crystals of *potassium nitrate* until no more gas is liberated, tipping the crucible from side to side to insure mixing. Distribute the melt while cooling by turning the crucible.

Extraction. Place in a 600-ml. beaker, cover with water, and heat below boiling for a short time. Allow to stand overnight. Remove crucible and cover, rinsing well, add 10

ml. of *85% phosphoric acid* to neutralize part of the alkali, and heat on the steam bath for 3 or 4 hours with occasional stirring. Cool, filter, wash with cold water up to 550 to 600 ml. into an 800-ml. beaker. Destroy nitrites by adding to the clear and colorless solution 10 ml. of *20% sodium bisulfite solution*, and heat just to boiling. When cool, add 30 ml. of *85% phosphoric acid* from a buret, continuing the addition rapidly until neutral to *methyl orange*, then 1.5 ml. in excess. Add a piece of anthracite coal (0.5 cm.), boil for 20 minutes or longer down to 400 to 500 ml. to remove all traces of sulfurous acid, cool, add *bromine water* to a permanent yellow color, then boil until colorless by reflected light and for 5 minutes longer. Add a few crystals of *salicylic acid* to remove the last traces of bromine, cool, then add 5 ml. of *20% reduced phosphoric acid solution*, and 0.5 to 1 g. of *potassium iodide*.

Titration. Proceed as in the original Kendall method.

NOTE. See also Determination of Iodine in iodized salt, Part II, L.

II. Pfeiffer Wet Combustion Modification.[110] The distinctive feature of this method (Bonn University) is the digestion with sulfuric acid and an oxidizing agent analogous to the wet combustion method for copper determination and the digestion in the Kjeldahl process. The method is claimed to be especially efficient in the oxidation of fats and oil seeds.

APPARATUS. The oxidation is carried out in a special tubulated flask connected with an electric- or gas-heated quartz tube, containing a platinum gauze coil, and this in turn with two absorption bottles.

The *Tubulated Flask*, ordinarily of 1-liter capacity, has attached to it by a ground joint a *Reagent Reservoir* with a stopcock, two bulbs, and a side tube connected with a *Wash Bottle*. The outlet tube of the flask is joined by a rubber connection with the *Quartz Tube*, 7 to 8 mm. in diameter and 14 cm. long except for the constrictions at the ends, and this in turn by a double-bent tube 24 cm. long, with the first of the two *Absorption Bottles*. The second absorption bottle is connected with a horizontal tube containing pieces of unglazed pottery moistened with *5 to 10% potassium hydroxide solution*.

For quantities of the sample exceeding 10 g. of dry matter, 1.5- to 2-liter flasks and a quartz tube of double diameter are needed.

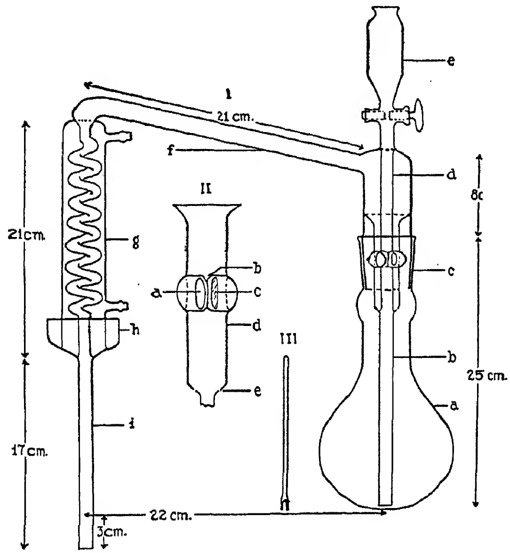
In order to prevent a too rapid evolution of carbon dioxide from samples of oils and fats, a 5- to 10-g. charge is introduced in 0.5- to 1-g. portions from a pipet, which temporarily replaces the stopper of the flask.

Heating the platinum gauze to redness destroys volatile fatty acids and the absorption bottles, containing respectively 5 and 1 or 2 ml. of saturated potassium carbonate solution diluted with 5 ml. of water, absorb iodine vapors. If more than 1 or 2 γ of iodine per milliliter of absorption fluid is expected, potassium hydroxide solution is used instead of potassium carbonate.

PROCESS. *Combustion.* Regulate the amounts of the sample, the sulfuric acid, and the hydrogen peroxide according to the kind and water content of material and the iodine content. Usually 10 g. of dry material, 150 ml. of *sulfuric acid*, and several 1- to 2-ml. portions of *30% hydrogen peroxide solution*, added dropwise, suffice.

Moisten the material thoroughly with a portion of the *sulfuric acid* and add the remainder slowly in small portions with shaking, avoiding the formation of lumps of charred matter. Before heating, add 20 to 40 drops of *30% hydrogen peroxide solution* with shaking and heat the quartz tube to redness to insure burning of ready-formed volatile substances. Start a steady flow of air through the train and cautiously heat to 200° (this usually requires 3 to 4 minutes and for double amounts of sample and reagents about 5 minutes). Reduce the air current when burning begins. Add *hydrogen peroxide solu-*

tion as required, keeping a steady evolution of carbon dioxide as indicated by comparing the rate of bubbling through the absorption flasks with that of air through the wash bottle at the right. A too vigorous action is checked by stopping the addition of hydrogen peroxide. If unburned carbon accumulates in the quartz tube, increase the amount of *peroxide* and remove the flame for a time.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1938, **10**, 613

FIG. 70. Matthews, Curtis, and Brode Iodine Distillation Assembly.

As is true in both wet and dry combustion, the operator must be guided by his own experience in regulating the size of the apparatus parts, the amount of the sample and reagents, and the rate of gas evolution.

Titration. Make up the combined solutions from the absorption flasks, which should not exceed 150 to 200 ml., to a definite volume in a volumetric flask and remove 10 ml. to a 50-ml. Erlenmeyer flask. Acidify with *1 + 1 hydrochloric acid,* add freshly prepared *chlorine water* until the liquid smells strongly of the gas. If much hydrogen sulfide is evident, add more *chlorine water* and boil vigorously for 3 to 4 minutes. Finally add a few drops of *0.2% starch solution,* a few small crystals of *potassium iodide,* and titrate with a *standard 0.002 or 0.0001 N sodium thiosulfate solution* made up daily.

Leipert Chromic Acid-Arsenic Trioxide Volumetric Method.[111] In this method, designed for biological material (blood, urine, thyroid, cod liver oil), the organic matter is oxidized by *chromic acid* and *sulfuric acid* catalyzed by *cerium sulfate* [$Ce(SO_4)_2$], the excess of chromic acid and the iodine pentoxide is reduced by a solution of *arsenic trioxide* (As_2O_3) in boiling *potassium hydroxide solution,* and the liberated iodine is distilled with steam into *20% sodium hydroxide solution* and titrated.

I. Trevorrow and Fashena Dichromate-Phosphorous Acid Modification.[112] In addition to substituting *potassium dichromate* for chromic acid and *phosphorous acid* for arsenic trioxide, heating at 195° to remove acetic acid and destroy chromium oxychloride is interposed between the oxidation and reduction.

II. Matthews, Curtis, and Brode Modification.[113] Methods of purification of the reagents have been developed at Ohio State University. By the use of a special distilling apparatus, vacuum-steam distillation and aeration distillation are unnecessary.

APPARATUS. The *Distillation Assembly* (Fig. 70) is supplied by The Leonard Glass Works, Columbus, Ohio. The part that needs explanation is the entrainment trap (II). Vapors enter at *a*, follow the concentric tube around *d*, and enter *d* through *c*. The concentric tube is closed off at *b*. Three small holes are located at *e*. III is an anti-bump made by sealing a 3-mm. glass rod onto a glass tube of 3 mm. inside diameter and cutting off the tube at 5 mm. and the rod to a suitable length.

REAGENTS. *Double Distilled Water.* Make the second distillation with 1% KOH solution.

Sulfuric Acid, iodine-free. Mix 10 ml. of HCl with 1 liter of technical H_2SO_4 and boil slowly for 30 minutes.

Chromium Trioxide Solution, 10 *M.* Heat in an oven at 160° in separate flasks 1 mole of large crystals of recrystallized potassium dichromate and 3.3 moles of H_2SO_4. Pour the acid quickly on the crystals, stir the violently reacting mixture, in which the CrO_3 liquifies, until the temperature drops to 175°, then pour off the H_2SO_4 solution and wash the solid CrO_3 with 0.5 mole of 65% H_2SO_4. Obtain the yield (80 to 90%) of the CrO_3 by subtracting the weight of the flask alone from that of the flask plus contents. Add 0.6 ml. of water for each gram of chromium trioxide.

Phosphorous Acid Solution, 5 *M.* Add 1 part of P_2O_3 in small amounts to 1.5 parts of continuously cooled water. When the reaction is complete, boil first in the air at 150°, then under reduced pressure at 180°; 1 ml. = 0.41 g. of anhydrous H_3PO_3.

Sulfurous Acid Solution, 0.1 *M.* Weigh 11 g. of $NaHSO_3$ into 1 liter of water. Add sufficient dilute H_2SO_4 through the entry tube to decompose the bisulfite and distil. The solution decomposes readily.

Potassium Permanganate Solution, 0.2 *M;* 1 ml. = 0.0316 g. of K_2MnO_4.

Sodium Nitrite Solution, 1.5 *M;* 1 ml. = 0.104 g.

Starch Indicator. Make 10 g. of soluble starch into a paste, add 10 mg. of powdered HgI_2 and 1 liter of boiling water, then boil for 5 minutes.

Urea Solution, 5 *M;* 1 ml. = 0.3 g.

Potassium Iodide Solution, 0.3 *M;* 1 ml. = 0.0498 g. Cool to 0° and test with colorless starch indicator.

Standard Sodium Thiosulfate Solution, 0.1 *N.* Dissolve 24.82 g. of the $Na_2S_2O_3 \cdot 5H_2O$ in water and dilute to 1 liter. Add 0.5 ml. of NH_4OH as a preservative. Standardize weekly against standard 0.1 *N* potassium biniodate (32.51 g. per liter). Store in a dark place. Dilute to 0.0002 or 0.001 *N* as needed.

PROCESS. *Digestion.* Oxidize dried foods and other material of low iodine content as directed by von Kolnitz and Remington (see McClendon-Remington Closed Combustion Method above). Boil down the basic absorbing solution and washings to 10 ml. and treat with 10 ml. of *10 M chromium trioxide solution* and *50 ml. of sulfuric acid.* Use for 25 ml. of milk 30 ml. of *chromium trioxide solution.* After the addition of the sulfuric acid, heat rapidly to 220° and maintain that temperature for 5 minutes.

Distillation. When the solution has cooled below 100°, add water equivalent to 25 ml., in excess of the sulfuric acid employed, and two anti-bumps, then mix by rotating at a 45° angle. Connect with the distillation apparatus. Place an Erlenmeyer flask, containing an anti-bump, 0.5 ml. of *0.5 M potassium carbonate solution* and 0.5 ml. of *0.1 M sulfurous acid,* under the condenser so that the tip of the stem dips beneath the surface. Heat the flask and when distillation begins add through the entry tube 5 ml. of *5 M phosphorous acid* for each 100 ml. of sulfuric acid in the flask. Conduct the distillation to yield 100 ml. or less of distillate in 15 to 20 minutes. Boil down the distillate to 3 to 5 ml., transfer to a 25-ml. Erlenmeyer flask, and concentrate to about 1 ml.

Oxidation. Heat the Erlenmeyer flask in a boiling water bath, add 1 drop (0.03 ml.) or 2 of *0.2 M potassium permanganate solution* directly into the liquid to a permanent purple color, gently rotate the flask, and heat for 2 minutes, then add 2 drops (0.06 ml.) of *85% phosphoric acid* and let stand for 2 minutes. To the solution (which should not be decolorized), add *1.5 M sodium nitrite solution* dropwise until all manganese dioxide and excess of permanganate are reduced, then 1

drop in excess and rotate the flask so that the solution rinses down the sides. Let stand 2 minutes, then add 1 drop of *5 M urea solution* and again mix by rotating. Heat 4 minutes longer in the water bath and cool.

Titration. Add 1 drop each of *0.3 M potassium iodide solution* and of *starch indicator* and titrate with *0.0002 or 0.001 N sodium thiosulfate solution.*

NOTE. If more than 20 γ of iodine is present in the charge, increase the amounts of the reagents used in the distillation and titration, also increase the volume of the solution to which the permanganate is added.

Other Methods. Schwaibold [114] describes a volumetric method with no new features. Sadusk and Ball [115] endorse the Winkler method.[116]

McCullagh [117] heats with *potassium hydroxide solution* in an open dish, then burns the ethanol extract in a stream of *oxygen*, finally employing both *hydrogen peroxide* and *bromine* as oxidizing agents. Elek and Harte [118] advocate *peroxide* fusion in the Elek-Hill micro bomb. Harvey [119] found that *hydrazine sulfate* reduces iodate. Hamilton [120] demonstrated that the reaction is quantitative and that the alkaline solution of iodate should not be boiled in porcelain dishes. On these data, he bases an improved method of extraction.

IRON

See also Part II, A2 and G1.

Iron is often stressed as the element most essential for animal and plant life. It is a constituent of the hemoglobin molecule and is present in all plants, although, contrary to the former view, it is now known that magnesium, not iron, is an integral part of the chlorophyl molecule.

As determined by modern methods, the amount of iron present in vegetable foods, even in roots and leaves where the contamination with soil may be considerable, usually is less than 1%, ash basis.

Unlike its sister metal aluminum, iron lends itself to colorimetric analysis because of the variety and intensity of the colored salts. Not only are the methods simple and delicate, but some are also selective in that the ferrous and ferric forms may be sharply differentiated by oxidation or reduction, even in the same weighed portion.

In the Scheme for Ash Analysis (Part I, C8a), iron and aluminum phosphates are weighed together, then dissolved in acid, and iron is determined by titration with standard potassium permanganate solution, and aluminum is obtained by difference. For many purposes this procedure is too lengthy and not sufficiently accurate for small amounts.

Marguerite-Zimmermann-Reinhardt Permanganate Volumetric Method. Kessler [121] and Zimmermann [122] showed that the high results obtained by the permanganate titration in hydrochloric acid solution may be corrected by the addition of a manganous salt and Reinhardt [123] that the end-point of the titration may be made sharper by the presence of phosphoric acid. The usual method is described by Treadwell-Hall.[124]

The modification described herewith has been used by Sherman [125] and others.

REAGENT. *Manganous Sulfate-Phosphoric Acid Reagent.* Dissolve 67 g. of $MnSO_4 + 4H_2O$ in 500 ml. of water with the addition of 138 ml. of H_3PO_4 (sp.gr. 1.7) and 130 ml. of H_2SO_4, then dilute to 1 liter.

PROCESS. *Incineration, Solution, and Reduction.* Burn 25 to 100 g. of the sample in a platinum or porcelain dish, dissolve in *hydrochloric acid*, and transfer the solution to a 200-ml. flask. Reduce the iron to the ferrous form with *hydrogen sulfide gas* as described in the Scheme for Ash Analysis; see Scheme, C8a, above.

Titration. Add 10 ml. of the *manganous sulfate-phosphoric acid reagent,* dilute to 100 ml., and titrate slowly, a drop a second, with *standard potassium permanganate solution* delivered from a micro buret.

Knecht and Hibbert Titanous Trichloride Volumetric Method.[126] The ferric chloride is reduced to the ferrous form by titanous chloride with the formation of titanic tetrachloride.

Reagents. *Standard Titanous Trichloride Solution*, 0.02 *N*. Keep in the dark in well-filled bottles and standardize against a solution of standard iron just before use.

Standard Iron Solution. See foregoing method.

Process. *Incineration and Solution*. See foregoing method.

Oxidation. If the iron is not in the ferric form or has not been so changed by boiling with hydrochloric acid together with nitric acid sufficient for oxidation, add *10% potassium permanganate solution* as directed in the Official Method until a very faint permanganate color persists.

Titration. Add 5 ml. of *10% thiocyanate solution* and titrate with *0.02 N titanous trichloride solution* to the disappearance of the red color.

Herapath Thiocyanate Colorimetric Method.[127] Although both potassium (or ammonium) thiocyanate and potassium ferrocyanide give color reactions suited for color comparison, the former is preferred by Walker[128] and several other authors. Color comparison made directly on the solution of the ash is inaccurate because of the presence of phosphoric acid which must be removed as in the following modification (University of Wisconsin).

I. Peterson and Elvehjem Modification.[129] Reagents. *Standard Iron Solution*. Dissolve 0.7 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (dried to constant weight over H_2SO_4) in 100 ml. of water, add 5 ml. of H_2SO_4, warm slightly, add $KMnO_4$ until the iron is oxidized, and dilute to exactly 1 liter; 1 ml. = 0.1 mg. of iron.

Potassium Hydroxide Solution, 40 and 2%. Allow to stand several days and decant off the iron-free top portion.

Process. *Incineration, Solution, and Phosphoric Acid Removal*. Reduce to ash a suitable amount of the sample in a platinum or acid-washed porcelain dish and take up in *1 + 2 hydrochloric acid*. Filter, keeping down the bulk, add *ammonium hydroxide* until a precipitate forms, then *iron-free nitric acid* until the precipitate dissolves. Heat, add *ammonium molybdate solution* in excess, allow to settle, filter off the ammonium phosphomolybdate, and wash with *9 + 100 nitric acid*.

Separation as Hydroxide. Heat the filtrate nearly to boiling, add *40% potassium hydroxide solution* until no more precipitate forms (usually about 20 ml.), boil several minutes to remove free ammonia, and cool. If the precipitate does not settle well, add more *potassium hydroxide solution* and heat again. Filter on a Gooch crucible (which with its mat of asbestos has been treated with hot hydrochloric acid) and wash the precipitate with *2% potassium hydroxide solution*.

Color Formation. Dissolve the precipitate in 2.5 ml. of *hydrochloric acid*, added dropwise to the crucible, and wash after each addition with hot water, keeping the volume of the filtrate down to 30 to 35 ml. Add *0.2 N potassium permanganate solution* until the filtrate is a faint pink (1 to 2 drops) and 5 ml. of *10% potassium thiocyanate solution*, then make up to 50 ml.

Color Comparison. Make the comparison against a solution containing 1 ml. of *standard iron solution* and the same amounts of reagents as were used in the solution of the unknown made up to 50 ml., or with a series of such solutions with different amounts of iron.

Examples. Wheat flour 9.1, salmon 34.4, peaches 28.5, almonds 42.3, potatoes, 38.1, and head lettuce 12.6 γ/g., dry basis.

II. Winter Modification.[130] The directions which follow are essentially those of the present Official Method, except as regards the incineration and preparation of solution.

REAGENT. *Standard Iron Solution.* Dissolve pure iron wire in HCl, with the addition of HNO_3 to oxidize to the ferric form, as in the actual analysis.

PROCESS. *Incineration.* Reduce a suitable amount of the substance to ash in a platinum dish.

Solution. Boil the ash with *hydrochloric acid* until all the iron has dissolved. In the experience of the writers ferric salts after incineration are not quickly dissolved, hence it is essential to repeat the boiling of the insoluble residue with *acid* after filtering until all iron has been removed. Filter, evaporate to dryness, separate silica if necessary, and make up to 200 ml.

Color Formation. Adjust an aliquot of the solution so as to contain about 0.2 mg. of iron, add water to about 40 ml. and 5 ml. of *hydrochloric acid,* then add 0.3 ml. of *nitric acid,* and boil 30 minutes. Transfer the solution to a 50-ml. volumetric flask, dilute to about 35 ml., cool, add 10 ml. of *20% potassium thiocyanate solution,* and fill to the mark.

Color Comparison. Make the comparison with a standard iron solution, containing about the same amount of iron as the unknown, treated in like manner.

Maquenne Ferrocyanide Colorimetric Method.[131] Several authors have taken advantage of the blue color formed by potassium ferrocyanide acting on ferric salts. Klut [132] used this reaction in water analysis, precipitating the iron with ammonium hydroxide, collecting and washing the ferric hydroxide on a filter, and dissolving in hydrochloric acid. Since the presence of phosphate precludes precipitation as hydroxide, Maquenne separates the iron as phosphate, following essentially the method used in the Scheme for Ash Analysis, Part I, C8*a*. A direct colorimetric comparison is inaccurate, partly because of the presence of copper.

APPARATUS. *Colorimeter.*

PROCESS. *Incineration and Solution.* Reduce to ash at dull redness 25 to 100 g. of the sample so prepared as to avoid contact with iron utensils. Moisten with *nitric acid* and again heat to destroy all carbon that would impart color to the solution. Add 1 ml. of *10% sulfuric acid,* evaporate cautiously until fumes appear, then heat with 3 drops of *hydrochloric acid,* dilute with 1 ml. of water, and decant into a 4-ml. centrifuge tube.

Separation as Phosphate. Whirl to cause any suspended calcium sulfate to settle. Decant off the clear liquid, add to it a few drops of *disodium phosphate solution* and *ammonium hydroxide* until a permanent precipitate is formed, then add *acetic acid* sufficient to dissolve any precipitate other than ferric phosphate. Whirl the solution and discard the clear supernatant liquid, after it has been shown by testing with *potassium ferrocyanide solution* to be free from iron.

Color Comparison. Dissolve the precipitate in 3 drops of *hydrochloric acid,* dilute with 2 ml. of water, and transfer to a centrifuge tube containing a few drops of freshly prepared *potassium ferrocyanide solution.* Compare the color in a colorimeter with that of a solution containing the reagents in a measured amount of *standard iron solution* or match with one of a series of standard solutions. Correct for any iron found in reagents, residues, and precipitates.

Lyons Thioglycolic Acid Colorimetric Method.[133] The red color produced when thioglycolic (mercaptoacetic) acid, mixed with a solution of iron, is made faintly alkaline was known to Andreasch [134] and Claësson [135] and may have been applied earlier. The reactions as given by Lyons (Parke, Davis and Co., Detroit) are

$$(1)\quad 2Fe^{+++} + 2HSCH_2COOH \rightarrow 2Fe^{++} + \begin{array}{l} SCH_2COOH \\ | \\ SCH_2COOH \end{array}$$

$$(2)\quad Fe^{++} + 2(SCH_2COOH^{-} \rightarrow \underset{\text{Colorless}}{Fe(SCH_2COOH)_2} \rightarrow \underset{\text{Colored}}{Fe(SCH_2COOH)_2^{--}}$$

Both ferric and ferrous iron may be determined in the same solution.

PROCESS. A. TOTAL IRON. Adjust the solution or an aliquot to about 1 : 500,000 as estimated from the color obtained in a qualitative test. Place 5 ml. in a test tube, add 1 drop of *thioglycolic acid* and 0.5 ml. of *ammonium hydroxide*. Treat in like manner 5 ml. of the *standard solution*.

CALCULATION. Obtain the gammas per gram (G) by the following formula:

$$G \quad \frac{10{,}000\,(100 \times U/S)}{Q}$$

in which U is the dilution of the unknown, S is the dilution of the standard, and Q is grams or milliliters used.

B. FERRIC IRON. In a new portion, determine ferric iron by the thiocyanate method (above). If the sample of the unknown is limited, make the determination of ferric iron first, add to the solution a few drops of *ammonium hydroxide* to discharge the color, and neutralize the ammonium hydroxide with *hydrochloric acid*, then add a drop of *thioglycolic acid* and proceed as directed under A.

C. FERROUS IRON. Subtract from the amount of total iron that of ferric iron.

LEAD

See also Part II, E5.

Vegetable and animal foods, produced under normal conditions, do not contain lead detectable by the usual chemical methods; spectrographic examination, however, may show exceedingly minute traces. Excepting possibly the coloring of confectionery with lead salts, long since discontinued, the presence of lead in foods is not intentional. Although formerly canned goods sometimes contained traces of lead derived from lead-containing tin plate or solder, it is probable that no American canner, or foreign canner shipping to the United States, today intentionally uses such materials. Again, the small amounts that may be derived from tanks, utensils, and containers are distinctly accidental impurities, the occurrence of which, if known, is soon corrected.

Spray residues on fruits and vegetables, that escape removal by dipping in an acid bath or other treatment, may be classed as accidental contamination.

Fischer Dithizone Colorimetric Method. Hellmut Fischer pioneered the application of the keto form of dithizone or diphenylthiocarbazone ($C_6H_5N{:}N\cdot CS\cdot NH\cdot NHC_6H_5$) in the determination not only of lead but also of zinc, copper, and other metals.

Dithizone is soluble in chloroform, carbon tetrachloride, and other organic solvents, also in ammonia, but not in weak acid solutions. The complexes with the metals differ in color and the optimum *p*H for reactions, but not so greatly in solubility, hence the need of preliminary separation.

I. Fischer and Leopoldi Modification. See Zinc below.

II. Clifford, Wichmann, Hubbard, and Bambach Modification. [136] APPARATUS. *Hellige Colorimeter*, *Neutral Wedge Photometer*, or *Comparison Tubes*.

REAGENTS. *Ammonium Citrate Solution.* Dissolve 40 g. of citric acid in water, add NH_4OH to alkaline reaction, as shown by phenol red indicator, and dilute to 100 ml. Delead by shaking with dithizone in chloroform.

Hydroxylamine Hydrochloride Solution, lead-free. Dissolve 20 g. of $NH_2OH\cdot HCl$ in water, dilute to about 65 ml., add a few drops of *m*-cresol purple indicator, and then add NH_4OH to a yellow color. Add 4% sodium diethyldithiocarbamate solution sufficient to combine with all the lead and most other metals, leaving a considerable excess. Extract the organo-metallic complexes and the excess of the reagent with chloroform, as shown by the absence of a yellow color when a portion of the chloroform is shaken with a dilute solution of a copper salt. Add redis-

tilled HCl to the hydroxylamine hydrochloride solution until the indicator turns pink, then dilute with redistilled water to 100 ml.

Potassium Cyanide Solution, 10%, lead-free. Dissolve 50 g. of KCN in water and make up to 100 ml. Shake with several portions of dithizone in chloroform (30 γ/ml.) until the lead is removed. Dilute to 500 ml.

Reclamation of Chloroform. Biddle [137] proceeds as follows. Remove the aqueous layer, wash until colorless with 5 to 10% its volume of H_2SO_4, treat with lime, distil in the presence of an excess of lime, and add ethanol to 1 to 1.5% of the volume as a preservative.

Hubbard further purifies the reclaimed chloroform as follows. Shake 1 liter in a large separatory funnel with 100 ml. of 0.5% $NH_2OH \cdot HCl$ solution, neutralized with 1% NH_4OH to *p*H 7.5 as shown by phenol red indicator. Filter through a dry pleated paper to remove water. The solution keeps for at least 2 months.

Dithizone Reagent. Dissolve 30 mg. of 86% dithizone in 1 liter of freshly reclaimed chloroform.

Fischer removes yellow impurities by first dissolving the dithizone in CCl_4 and shaking out with very dilute NH_4OH and second by acidifying the aqueous solution and shaking out the dithizone with CCl_4. Chloroform may be substituted for CCl_4.

Alkaline Ammonium Cyanide Solution. Dissolve 20 g. of NH_4CN in 150 ml. of redistilled NH_4OH and dilute to 1 liter.

Standard Lead Solution. To prepare a stock solution, dissolve 0.160 g. of $Pb(NO_3)_2$ (dried at 100°) in water and dilute to 1 liter; 1 ml. = 0.1 mg. of lead.

PROCESS. *Solution.* Ashing or wet combustion with *sulfuric acid* and *nitric acid,* with the addition during the heating of *perchloric acid* or *potassium chlorate,* is regarded as essential when the lead is disseminated throughout the mass of food products such as saccharine substances and the drained solids of canned goods. Baking chemicals and the liquor of canned goods may or may not require a similar preliminary destruction of organic matter, although at a subsequent stage ignition at a low heat may be required to remove organic matter that would interfere with colorimetric determination.

Since dipping in weak acid is a well-approved method of removal of spray residue employed by the orchardist, the same solvent seems the logical one for the chemist to use. A procedure adopted by the A.O.A.C. consists in dipping apples or pears first in a boiling solution containing about 3% of *sodium hydroxide solution* and 2.5% of *sodium oleate* and washing with hot *1.5% nitric acid* or *1% hydrochloric acid.*

Adjustment of Solution (Hubbard). Pipet into a separatory funnel an aliquot of the solution, representing one-tenth or other suitable fraction of the original portion, and from 0 to 100 γ of lead. Add 15 ml. of *purified ammonium citrate solution,* equivalent to 6 g. of monohydrous citric acid, (also as recommended by Bambach 1 ml. of *deleaded hydroxylamine hydrochloride solution*), 2 drops of *phenol red indicator,* and, dropwise with swirling, *redistilled ammonium hydroxide* to a distinct yellow color. Next add 5 ml. of *10% potassium cyanide solution,* and adjust to *p*H 7.5 with *ammonium hydroxide.*

Dithizone Extraction (Bambach). Extract the lead with 10 ml. of the *dithizone reagent,* added in two 5-ml. portions with shaking before drawing off the dithizone layer into another separatory funnel. Note the amount of lead removed as indicated by the color of the dithizone extract which ranges from bright red through purple to bluish green in descending order. Make other extractions if necessary, but usually the 10-ml. extract contains the larger part of the lead.

Chloroform Extraction (Bambach). Wash the combined dithizone extract, containing a certain amount of ammonium hydroxide and

salts, with 50 ml. of water, then extract the last with 5 ml. of *pure chloroform.* If the chloroform extract is green, owing to the presence of lead, extract further the water solution with another 5-ml. portion of *chloroform.*

Nitric Acid Extraction (Bambach). Add the chloroform extract to the dithizone solution and shake with 50 ml. of *1 + 100 nitric acid.* Drain off the dithizone solution to 0.5 ml. and reject. Add 3 drops of *m-cresol purple indicator* to the nitric acid solution, then add *dilute ammonium hydroxide* to an orange color (pH 2) and shake vigorously. The absence of color change in the 0.5 ml. dithizone solution is proof of the absence of bismuth which, if present (as in certain biological preparations), may be removed by further shaking with the *solvent.*

Drain off the dithizone, add 5 ml. of *chloroform* to the aqueous layer, shake, and allow to stand unstoppered until the drop of chloroform floating on the surface of the dilute nitric acid has evaporated; then draw off the chloroform as completely as possible, guarding against the passage of more than a drop of the aqueous solution into the stopcock, and discard. Proceed with the aqueous solution as follows.

Color Formation.[138] Bambach directs as follows: To the separatory funnel containing the aqueous solution of the lead, add 10 to 25 ml. of *dithizone reagent* and 7 ml. of *alkaline ammonium cyanide solution* and shake 1 minute, taking care not to release the pressure through the stopcock. Draw off the dithizone solution, using the first portion to flush the stem, and make the color comparison.

Color Reading. Fischer and Leopoldi make the colorimetric determination in a Hellige colorimeter, Clifford and Wichmann, Hubbard, and Bambach in a neutral wedge photometer. Wichmann[139] states that in 16 U. S. Food and Drug Administration Laboratories homemade neutral wedge photometers have given good service and that a form devised by Clifford and Brice is being made commercially.

Colorimetric comparisons may, however, be made in the usual manner with *standard lead solutions* treated like the solution of the unknown.

III. General A.O.A.C. Scheme. Details (15 pages) tentatively adopted by the Association and published in *Methods of Analysis*, appear under the following heads: (1) ashing, (2) separation of lead either as the dithizone complex or the sulfide, (3) electrolytic or colorimetric dithizone determination in comparison tubes or with a photometer, (4) interference, and (5) special methods. Much of the data given under the Fischer method and the foregoing modification are covered by this general scheme.

IV. Winter, Robinson, Lamb, and Miller Modification.[140] The modification was developed at the Michigan Agricultural Experiment Station for the determination of lead in spray residues.

Apparatus. *Colorimeter.*

Reagents. *Cyanide-Citrate Reagent.* Add 30 ml. of 5% KCN solution, 15 ml. of 5% $(NH_4)_2HC_6H_5O_7$ solution ($C_6H_8O_7 \cdot H_2O$ made slightly alkaline with NH_4OH), and 5 ml. of NH_4OH to 450 ml. of distilled water.

Alkaline Cyanide Reagent. Add 10 ml. of 5% KCN solution and 5 ml. of NH_4OH to 500 ml. of distilled water.

Test the last two reagents as follows. To 20 ml. in a small separatory funnel, add 1 ml. of dithizone solution and 5 ml. of chloroform, shake, separate, and extract the chloroform layer once or twice with another 20 ml. of the solution. The final chloroform layer should be colorless or very faintly red if separatory funnels made from ordinary glass are used. Also run a blank determination on the reagents as used in the analysis.

If necessary, purify as follows. Shake vigorously for 5 minutes the cyanide-citrate reagent with a few milliliters of dithizone so-

lution and 15 ml. of chloroform. Allow to separate at least 5 minutes. If the chloroform layer is red and the water colorless, add more dithizone and repeat. Draw off the chloroform layer; repeat the treatment, adding about 1 ml. of dithizone and 10 ml. of chloroform each time until the chloroform layer is colorless or slightly green. This layer should become colorless or very faintly red when extracted once or twice with the alkaline cyanide reagent.

Treat the alkaline cyanide reagent in like manner, but add no more dithizone than is necessary to remove all the lead. The water layer may have a slightly brownish color immediately after preparation but should become entirely colorless after standing several hours. The solution should not be used until it is colorless. The brown color may be removed by shaking out with 0.25 g. of ash-free decolorizing charcoal.

Dithizone Solution. Dissolve 10 mg. in 400 ml. of chloroform.

Standard Lead Solution. Dissolve 1.5985 g. of recrystallized $Pb(NO_3)_2$ in 100 ml. of 0.1% HNO_3; 1 ml. = 0.01 g. of lead. Dilute 0.5 ml. of this stock solution to 500 ml; 1 ml. = 0.01 mg. of lead.

PROCESS. *Removal of Spray Residue. A. Wichmann and Vorhes Procedure.*[141] Impale each apple on a pointed glass rod, swirl in boiling *sodium oleate-sodium hydroxide solution* for 1 minute or until the skin breaks, then wash with hot *1% acetic acid* delivered from a rubber bulb 25-ml. pipet. Combine the dipping and wash solutions and make up to 500 ml. with water. Add a 100-ml. aliquot to 10 ml. of *nitric acid* to precipitate soap and wax, filter, and remove 1 to 5 ml. with a pipet.

B. Wichmann aud Clifford Procedure.[142] Peel the fruit, digest an aliquot of the peelings with *nitric acid*, and dilute to 500 ml. Remove a suitable aliquot.

Extraction. To 15 ml. of the *cyanide-citrate reagent* in a 150-ml. separatory funnel, add the neutralized aliquot of the sample, then add with shaking from a buret *dithizone solution* in 1-ml. portions until, after vigorous shaking for a few seconds and settling, the lower layer is noticeably purple. Add *chloroform* from a buret, making the volume of chloroform plus dithizone exactly 10 ml. Shake with swirling and horizontal motions to dislodge drops of chloroform from the top of the aqueous layer. Draw off the chloroform layer into a second separatory funnel containing 20 ml. of the *alkaline cyanide reagent.*

Excess Dithizone Removal. Shake the second separatory funnel, allow to separate, and draw off the aqueous layer, repeating the extraction a second and, if necessary, a third time, using 20 ml. of the *alkaline cyanide reagent* until the upper solution is practically colorless.

Color Formation. Open the stopcock slightly until chloroform replaces the aqueous solution in the bore, then remove with a cotton swab the aqueous drops from the stem of the funnel. Draw off the clear or slightly turbid chloroform layer into a dry test tube, retaining in the separatory funnel any slight emulsion from between the two layers. Stopper the test tube and, if necessary, allow to stand for several hours for any cloudiness, due to an emulsion or cooling, to disappear.

Color Comparison. Compare in a colorimeter with a *standard solution* prepared in the same manner as the unknown. Since the solution does not obey Beer's law, prepare a series of standards containing 0.001, 0.0025, 0.0050, then to 0.04 mg. of lead in steps of 0.005 mg. and determine the color value against a solution containing 0.02 mg. of lead set at 20 mm.

V. Fabre and Lem Modification.[143] PROCESS. *Separation.* Dilute the solution, obtained by the wet combustion of 10 g. of the sample with *sulfuric* and *nitric acids*, with 30 ml. of water, add 5 ml. of *ammonium acetate*

solution and 10 ml. of *5% urea solution*, boil 5 minutes, cool, make faintly alkaline with *ammonium hydroxide*, and filter to remove ferric hydroxide. To the filtrate, add 5 ml. of *5% potassium cyanide solution*, *acetic acid* until acid, and *ammonium hydroxide* until faintly alkaline to *litmus*. The solution at this stage contains no metals other than lead capable of forming a stable compound with dithizone.

Color Formation. Shake out with several portions of *0.06% dithizone in carbon tetrachloride*, remove the excess of dithizone by shaking out with water, then with *1.6% ammonium hydroxide*, and finally remove the lead from the carbon tetrachloride solution by shaking out with *2 N hydrochloric acid*.

Color Comparison. Make the color comparison on the dithizone solution as described below.

VI. White Rapid Field Modification.[144] Dissolve the lead from the surface of fruit by washing 2 to 6 times with a solution of *dithizone in chloroform*, in the presence of an aqueous solution of potassium cyanide, ammonium hydroxide, and citric acid. Note the change of color of the dithizone indicative of lead, ignoring an occasional turbidity due to suspended matter. Contact with the fruit skin and the presence of other constituents of the spray do not appear to interfere with the test, but the low solvent action of the dithizone solution is a serious obstacle to making the test quantitative.

Letonoff and Reinhold Chromate-Diphenylcarbazide Colorimetric Method.[145] Although *diphenylcarbazide* ($C_6H_5 \cdot NH \cdot CO \cdot NH \cdot NH \cdot C_6H_5$)—not to be confused with diphenylthiocarbazone (dithizone)—has not been found to be a satisfactory reagent for lead analysis by several authors, a method based on its use has been devised at the Philadelphia General Hospital and School of Medicine of the University of Pennsylvania for the detection of lead poisoning. The method appears to be particularly adapted for the determination of lead in body fluids and excreta, although brief mention is made of tissues, bone, food, and water.

The steps in the process are: (1) ashing, (2) precipitation as lead potassium chromate in the presence of chloride, citrate, acetate, and ammonium ions at *p*H 6.6 to 7.4 in a centrifuge tube, followed by centrifuging and washing, (3) formation of a red color with diphenylcarbazide, and (4) color measurement in a photoelectric colorimeter or comparison with standard lead chromate treated with the carbazide in a colorimeter.

Kozelka and Kluchesky Photoelectric Colorimetric Method.[146] Full details of the method for the determination of lead will appear in a later publication from the University of Wisconsin. The method eliminates the necessity of removing the excess of dithizone and the use of standard dithizone solutions, permitting the photoelectric colorimetric determination of one component in a two-component system.

Zotova Cupric Nitrate Electrochemical-Colorimetric Method.[147] APPARATUS. *Electrolytic Assembly.*

REAGENT. *Nitrate Mixture.* Dissolve 40 g. of $Al(NO_3)_3 \cdot 9H_2O$ and 20 g. of $Ca(NO_3)_2 \cdot 4H_2O$ in 100 ml. of water.

PROCESS. *Incineration.* Add to 100 g. of the sample in a porcelain dish 5 ml. of the *nitrate mixture*, carbonize over a small flame, and burn to a white ash in a muffle furnace at 450 to 500°.

Solution. Dissolve the ash by boiling for 10 to 15 minutes with 10 ml. of *1 + 1 nitric acid* in a 300-ml. beaker, then add 5 ml. of *20% cupric nitrate solution* and 200 ml. of hot water.

Electrolysis. Heat on a hot water bath and electrolyze for 2 hours at 1.5 amperes and 5 to 6 volts, using a spiral platinum wire surrounding a glass stirrer as the cathode and Winkler's electrode (a wire-mesh cylinder) as the anode. Wash the anode and stop the current.

Resolution. Dissolve the lead dioxide from the anode with 10 to 15 ml. of *nitric acid* and 3 to 5 drops of *hydrogen peroxide*. Evaporate to dryness on the water bath and dissolve the residue in 0.5 ml. of *1 + 1 nitric acid.*

Colorimetric Determination. Use one of the foregoing methods.

Wilkins, Willoughby, Kraemer, and Smith Dithizone Titrametric Extraction Method.[148] This adaptation of the Fischer method is used in the laboratory of the Tumor Clinic of Jefferson Hospital, Philadelphia, for the determination of minute amounts of lead in blood, excreta, tissues, etc. One dithizone solution is employed in the separation of the lead from other metals and another in the final "quantitative titrametric extraction."

As carried out in standardizing the second dithizone solution, 5 to 20 ml. of *standard lead nitrate solution* (0.01 mg. of lead per milliliter), 2 ml. of *10% potassium cyanide solution*, and 2 to 3 drops of *phenol red indicator* are adjusted to pH 7.5 with *5% nitric acid*, a suitable volume of *dithizone solution* is added (10 ml. for 0.2 mg. of lead) and the mixture is vigorously shaken. The green color of the reagent solution is discharged with the formation of the brilliant reddish orange-colored lead-dithizone complex which is soluble in chloroform, but not appreciably in the aqueous phase. After settling, the chloroform layer is discarded, 5 ml. of the *dithizone solution* are added to the separatory funnel, and the extraction is repeated. This is repeated, smaller and smaller volumes of *dithizone solution* being used until finally, with a few drops of *chloroform* added, the reagent changes from green to purple. In the actual analysis, the titrametric extraction is carried out in the same ingenious and distinctive manner.

Cholak Spectrographic Method.[149] As first described by Cholak (University of Cincinnati), the method was designed only for urine, but later was adapted to the analysis of other biological materials.

A spectrograph with accessories and a Bausch & Lomb microphotometer are employed to measure the line intensities. Knowledge of the technique of emission spectrography is essential.[150]

Manganese

The methods long employed for ash analysis, although exact for the commoner constituents, either ignore manganese or group it with iron.

Examples of results by modern methods are the following: wheat 36 to 73, soy beans 21 to 41 γ/g. (McHargue); potatoes 6 to 14, asparagus 28 to 86, spinach 68 to 105 γ/g. (Remington and Shiver).

Several authors have taken advantage of the reactions whereby manganese salts are converted, through oxidation, notably by alkali, bismuthate, persulfate, or periodate, into permanganate which, by reason of the intense color of its solution, is well suited for colorimetric comparison.

Reddrop and Ramage Bismuthate Colorimetric Method.[151] Although now not so commonly used as formerly, this method, depending on sodium bismuthate as the oxidizing reagent, has been adapted with success by Gortner and Rost [152] for soil analysis. It was designed to replace the lead dioxide process suggested by Crum [153] and developed by Pichard.[154]

Apparatus. *Color Comparison Tubes.*

Process. *Incineration and Solution.* Prepare the ash and dissolve in *sulfuric acid* with the addition of *nitric acid.* Evaporate and heat to remove the hydrochloric and nitric acids, repeating if necessary.

Color Formation. Heat to boiling the dilute sulfuric acid solution, add 0.02 to 0.5 g. of *lead-free sodium bismuthate,* and continue the boiling until the full color is developed.

Color Comparison. Proceed with the colorimetric comparison with *standard manganese*

solution as in the Willard and Greathouse Method below.

NOTE. A method, employing bismuth subnitrate, was devised by Weston [155] for the determination of manganese in water.

Marshall-Walters Persulfate Colorimetric Method.[156] This method or its equivalent has been extensively used in mineralogical, metallurgical, and water analysis, also by Bertrand,[157] Haas,[158] and others in food analysis.

APPARATUS. *Color Comparison Tubes.*

REAGENTS. *Silver Nitrate Solution.* Dissolve 4.79 of $AgNO_3$ in water and make up to 1 liter; 1 ml. = 1 mg. of chlorine.

Standard Manganese Sulfate Solution. Dissolve 4.388 g. of $MnSO_4 \cdot 5H_2O$ in water and dilute to 1 liter; 1 ml. = 1 mg. of manganese.

PROCESS. *Incineration and Solution.* Burn to a white or gray ash 25 to 100 g. of the material in a porcelain or quartz dish. Add a small amount of water, 10 ml. of *sulfuric acid*, and a few milliliters of *nitric acid.* Heat until the ash, other than sand, has dissolved and filter. If the insoluble matter contains a considerable amount of carbon, burn with the paper and boil the ash with *sulfuric* and *nitric acids* as before; if the filtrate appears to contain organic matter, evaporate and burn at a low heat, and proceed as before.

Silver Precipitation and Persulfate Oxidation. Add *silver nitrate solution* until no more precipitate forms; then add a few milliliters more. Bring to a boil, add 1 to 2 g. of *potassium persulfate*, or a sufficient amount to bring out the maximum color, and continue the boiling 15 minutes, observing whether the color ceases to increase in intensity. Cool and make up to a definite volume.

Color Comparison. Prepare a solution containing a measured amount of the *standard manganese sulfate solution*, corresponding approximately to that in the unknown, together with the same amount of reagents and compare the colors as in the following method.

Willard and Greathouse Periodate Colorimetric Method.[159] It is believed that this method, developed at the University of Michigan, is free from certain faults of the long-used persulfate method, such as variable and fading color and incomplete oxidation. The reaction had previously been employed in qualitative tests, but not in colorimetric analysis:

$$2Mn(NO_3)_2 + 5KIO_4 + 3H_2O \rightarrow 2HMnO_4 + 5KIO_3 + 4HNO_3$$

The method has been applied to the determination of manganese in numerous foods by McHargue.[160]

I. Skinner and Peterson Modification.[161] The method was adapted at the University of Wisconsin for the small amounts in vegetable and animal products.

APPARATUS. *Color Comparison Tubes.*

REAGENT. *Standard Manganese Sulfate Solution.* Dissolve 2.877 g. of crystalline $KMnO_4$ in water, reduce in a stream of SO_2, expel the excess by boiling, and dilute to 1 liter; 1 ml. = 1 mg. of Mn.

PROCESS. *Incineration and Solution.* Incinerate a suitable amount in a porcelain dish in which has been heated *1 + 4 hydrochloric acid* to remove any contamination with manganese. Finish the ashing at a dull red heat in a muffle furnace. As first suggested by Davidson and Cohen, add to the ash 5 ml. of *sirupy phosphoric acid* and 30 to 50 ml. of water, heat on a water bath for about 30 minutes, cool, and filter through paper or manganese-free asbestos into a 250-ml. beaker.

If the material, such as olives, has a considerable content of chlorine, dissolve the ash, contained in a porcelain dish, in *dilute sulfuric acid* with the addition of 5 ml. of *nitric acid*, evaporate until fumes appear, repeating twice, then oxidize and proceed as above.

Conversion into Permanganate. Boil gently with about 0.3 g. of *potassium periodate* until

the conversion into potassium permanganate is complete as indicated by the color. Cool to about 40°, remove to a comparison tube, fill to the mark, and mix by pouring back and forth into the beaker.

Color Comparison. Without delay, compare with a *standard solution* prepared with the same amounts of reagents and containing approximately the same amount of manganese. It is recommended to prepare first 2 standard solutions from a standard stock solution of manganese sulfate, one darker, the other lighter, than the unknown and pour from the darker to the lighter until the color of the unknown is nearly matched. A once-folded sheet of white paper resting tent-like on another sheet forms a convenient reflector for illuminating the tubes. A millimeter rule serves quite as well as graduations on the tube for measuring the relative height of the columns.

If a cloudiness appears, evaporate to about 20 ml., allow to settle, decant cautiously, reoxidize, and compare with the standard.

Note. Richards [162] prefers the Willard and Greathouse periodate method to the Marshall-Walters persulfate method because no catalyst is used for converting Mn^{++} into MnO_4^-. The former yields good results when applied to animal and vegetable ash if all chloride is removed before the final oxidation and the acidity is kept within 15 ml. of sulfuric acid per 100.

II. Smith Modification.[163] The procedure in the form adopted for grain and stock feed follows.

Apparatus. *Colorimeter.*

Reagent. *Standard Potassium Permanganate.* Boil 1.4385 g. of $KMnO_4$ with water until dissolved, dilute to 1 liter, let stand several days, and filter through asbestos with suction. Standardize with sodium oxalate; 1 ml. = 0.5 mg. Pipet 40 ml. (= 20 mg. of Mn) into a beaker, add 100 ml. of water, 15 ml. of 85% H_3PO_4, and 0.3 g. of KIO_4, and bring to a boil. Cool and dilute to 1 liter; 1 ml. = 0.02 mg. of Mn. Protect from light. Prepare suitable standards by dilution with water that has been boiled with 0.3 g. of KIO_4 per liter.

Process. *Incineration and Solution.* Burn 5 g. of the sample at dull redness in a porcelain dish, cool, add 5 ml. each of *sulfuric acid* and *nitric acid*, and evaporate to white fumes. If carbon is not completely destroyed, add more *nitric acid*, boiling after each addition. Cool slightly, transfer to a 50-ml. volumetric flask, and add 25 ml. of *phosphoric acid* (8 ml. of 85% phosphoric acid plus 92 ml. of water). Again cool, make to volume, mix, and filter or let stand until clear.

Color Formation. Pipet 25 ml. of the solution into a beaker or directly into a 50- or 100-ml. volumetric flask, and add 15 ml. of water and 0.3 g. of *potassium periodate* for each 15 mg. of manganese present. Mix and heat below the boiling point for 30 minutes, or until the maximum color develops. Cool and dilute with water to an accurately measured volume, usually 50 or 100 ml.

Color Measurement. Compare with the *standard potassium permanganate solution* in a colorimeter.

Calculation. Express results as gammas per gram of manganese in the sample.

Sideris Formaldoxime Colorimetric Method.[164] In the study of manganese in soils and plants at the Pineapple Experiment Station of the University of Hawaii, the formaldoxime reagent of Denigès is employed.

Apparatus. *Colorimeter.*

Reagents. *Formaldoxime Reagent.* Dissolve by boiling 20 g. of trioxymethylene and 47 g. of hydroxylamine sulfate in 100 ml. of water.

Ferric Chloride Solution. Dissolve 0.4876 g. of $FeCl_3 \cdot 6H_2O$ in 1 liter of water and add 5 ml. of 0.077 *N* HCl; 1 ml. contains 0.100 mg. of Fe^{+++}. Deliver from a microburet.

Gum Ghatti Solution, 5%. Dissolve 5 g. of gum ghatti by boiling in 100 ml. of water.

Filter through cotton while warm and add 1 ml. of toluene for storage.

Standard Manganese Solutions. Dissolve 0.4061 g. of $MnSO_4 \cdot 4H_2O$ in 1 liter of water and add 1 ml. of H_2SO_4. From this stock solution, containing 0.100 mg. of Mn^{++} per milliliter, prepare solutions containing 0.002, 0.008, and 0.012 mg. per ml. of manganese and approximately the same amount of iron (as ferric chloride) as in the solution of the sample.

PROCESS. *Incineration.* Reduce to an ash a quantity of the sample containing 0.01 to 0.05 mg. or more of manganese, taking care that all the iron is oxidized to the ferric form. Dissolve the ash in 2 to 5 ml. of *0.077 N hydrochloric acid*, filter, wash with water, and dilute to 25 ml. in a volumetric flask. Dilute further if more than 0.02 mg. of manganese is present.

Determination of Iron. In an aliquot of the solution determine iron by Saywell and Cunningham *o*-Phenanthroline Method (see Part II, F1) or other reliable method.

Color Formation. To a 10-ml. aliquot of the acid solution of the sample and 10-ml. portions of each of the standards, add successively 0.25 ml. (about 5 drops) of *40% potassium hydroxide solution*, 0.5 ml. of *20% sodium cyanide solution*, and 3 drops of *formaldoxime reagent*, stirring after each addition. The wine red color appears at once. Add also 0.5 ml. of *5% gum ghatti solution* to prevent adsorption of the pigment to the colloidal suspension formed after the addition of the potassium hydroxide solution.

Color Reading. Allow the unknown and standards to stand 20 minutes, then compare in the colorimeter.

SELENIUM

The intensive study in recent years of the assimilation of selenium by plants from soils rich in that element and the toxic properties of such plants led naturally to the search for suitable methods of determination. The most successful methods depend on two reactions: (1) the yellow color formed with *hydroxylamine hydrochloride*, employed by Robinson and associates, of the U. S. Bureau of Chemistry and Soils, and made the chief feature of the method adopted tentatively by the A.O.A.C., and (2) the blue color formed with *codeine sulfate*, employed by Schmidt and later by Gortner and Lewis.

Robinson Hydroxylamine Colorimetric Method.[165] The four steps in the process are: (1) wet combustion with *sulfuric acid* and *nitric acid*, (2) distillation with *hydrobromic acid* and *bromine* with conversion into the hexavalent form, then into the quadrivalent, (3) precipitation with *hydroxylamine hydrochloride*, and (4) colorimetric determination in a *gum arabic solution*. Cousen[166] had previously used gum arabic for holding up the selenium compound, but he precipitated with *phenylhydrazine hydrochloride*.

The Robinson method was adapted for vegetable products by Robinson, Dudley, Williams, and Byers[167] and for animal products by Dudley and Byers.[168]

Iodometric Modification.[169] The novel feature is the substitution, for the color comparison, of iodometric titration as proposed by Norris and Fay,[170] depending on the following reaction:

$$H_2SeO_3 + 4Na_2S_2O_3 + 4HBr \longrightarrow$$
$$Na_2S_4SeO_6 + Na_2S_4O_6 + 4NaBr + 3H_2O$$

APPARATUS. *All Glass Distillation Assembly*, consisting of round-bottom flask, still head, thermometer, and condenser, as devised by Robinson *et al.*

REAGENTS. *Hydrobromic Acid-Bromine Reagent:* (*A*) *40%* containing 0.5% of bromine; (*B*) 40% containing 1% of bromine.

Standard Sodium Thiosulfate Solution, 0.01, 0.001, or 0.0005 *N*, equivalent respectively to 200, 20, and 10 γ of selenium per ml.

Standard Iodine Solution, corresponding in strength to the thiosulfate used.

PROCESS. *Sample.* Mix thoroughly in the distilling flask a quantity of the sample, containing 5 to 10 g. of dry matter, with a cooled mixture of 50 ml. of *sulfuric acid* and a volume of *nitric acid* equal to 10 ml. per gram of the sample, to which has been added 0.5 g. of *mercuric oxide.* After 30 minutes, heat gently until the evolution of nitric fumes ceases and the solution turns brown or sulfur trioxide fumes appear.

Isolation. Add 25 ml. of water to the cold digest, cool, transfer to the flask of the distilling assembly, rinsing with 50 to 60 ml. of *hydrobromic acid-bromine reagent A,* and distil into a mixture of 5 to 10 ml. of water and 2 to 5 ml. of the *reagent,* taking care that the adapter dips below the surface, until the temperature reaches 130°. Filter the distillate, if necessary, through asbestos, wash with 5 to 10 ml. of cold water, and saturate with *sulfur dioxide gas.* Add 0.1 g. of *hydroxylamine hydrochloride,* warm to 80° for 15 minutes, cool, filter through asbestos, and wash with 5 to 10 ml. of cold water.

Resolution. Estimate visually the amount of precipitated selenium on the filter and dissolve in 1 to 2 ml. of *hydrobromic acid-bromine reagent B,* using a few drops to rinse the precipitation flask. Wash with water to a total volume below 20 ml. (10 ml. for 20 γ or less of selenium).

Decolorization. Prepare standards and blanks, diluting to the same volume as the unknown and adding the same amounts of *reagents.* Add to unknown, standards, and blank a *saturated sulfurous acid solution* until the bromine color nearly disappears, adding more of the *hydrobromic acid-bromine reagent B* dropwise to a light yellow color if all the bromine is reduced. Decolorize with 1 to 2 drops of *5% aqueous phenol.*

Titration. To the solution in a 30- to 50-ml. beaker, add 1 ml. of freshly prepared *starch indicator,* then add rapidly from a 10-ml. buret, with delivery tip dipping into the liquid, *standard thiosulfate solution* in moderate excess, as estimated from the bulk of the precipitated selenium, plus 2 ml. additional. Employ a thiosulfate solution of such normality that the volume will be between 2 and 10 ml. After 20 to 30 seconds, add rapidly from a buret *standard iodine solution* to a permanent blue color. If the amount required is less than 1 ml., add 2 ml. of *thiosulfate,* then *iodine solution* until at least 1 ml. is needed to give a blue color. Complete the titration with *thiosulfate solution,* matching the color of the blank containing water and 1 ml. of *starch indicator.*

CALCULATION. *Blanks.* Divide the total milliliters of thiosulfate solution by the total milliliters of iodine solution to obtain the conversion factor for iodine to thiosulfate.

Standards. Multiply the milliliters of iodine solution by the factor and subtract the product from the total milliliters of thiosulfate solution and divide the remainder into the weight of selenium in the standard to obtain the gammas of selenium per milliliter of thiosulfate.

Unknown. Calculate the milliliters of thiosulfate solution used in the reduction of the selenium, as directed under Standards above, and multiply by the gammas of selenium per milliliter of thiosulfate solution, thus obtaining the total gammas of selenium which, divided by the total number of grams of the charge, gives the gammas per gram of selenium in the sample.

NOTE. Klein [171] has recently reported on further studies of the method.

Schmidt Codeine Sulfate Test.[172] As first devised, the solution of selenium was treated with *codeine phosphate.* The color obtained was green or bluish green. Considerable iron is stated to interfere with the reaction. As employed by Stover and Hopkins,[173] of the University of Illinois, dried leaves are digested with *nitric acid,* concentrated to small volume, again digested, and concentrated, then boiled with *sulfuric acid,* cooled, diluted, and tested with *codeine.*

I. Horn Quantitative Modification.[174] This modification was devised for plant experiments carried out in the U. S. Bureau of Chemistry and Soils.

Apparatus. *Bock Benedict Colorimeter.*

Reagent. *Standard Selenium Solution.* Dissolve 0.01 g. of metallic selenium in 50 ml. of H_2SO_4 at room temperature.

Process. *Solution.* Weigh 1 g. of the sample into a Kjeldahl flask, add 0.2 g. of *mercuric oxide,* and 40 ml. of *sulfuric acid* and digest until colorless. Cool and make up to a definite volume with the *acid.*

Codeine Treatment. To a 5-ml. aliquot, add 2 drops of *3% aqueous codeine sulfate solution,* cooling and shaking after each drop. The green color which forms rapidly changes to blue.

Color Comparison. Match the solution against 5 ml. of a *standard solution* containing 0.01 g. of elemental selenium in 50 ml. of sulfuric acid treated in like manner and set at 20 mm. in the colorimeter.

II. Gortner and Lewis Modification.[175] Fat extraction was found essential in the analysis of animal tissues, particularly liver, as carried out at the University of Michigan.

Apparatus. *Pulfrich Photometer* (Zeiss).

Process. *Fat Extraction.* Reflux a weighed amount of the ground fresh tissue or formalin-preserved tissue with 100 ml. of *chloroform* for 3 to 4 hours, filter, wash the residue twice with small portions of hot *chloroform,* and dry at room temperature.

Digestion. Weigh a suitable amount of the residue into a 100-ml. Kjeldahl flask, add 0.2 g. of *mercuric oxide,* rinse down the sides with 5 to 10 ml. of water, then add 40 ml. of *sulfuric acid* and 2 glass beads to prevent bumping. Digest for 6 to 9 hours, adding from time to time 5 to 10 drops of *30% hydrogen peroxide solution.* Cool, transfer the colorless solution to a 50-ml. volumetric flask, and dilute to volume with *sulfuric acid.* Mix and centrifuge for 15 to 20 minutes at 1800 r.p.m.

Color Formation. Pipet 10 ml. of the centrifugate into a clean, dry test tube, add 3 drops of *3% (saturated) aqueous codeine sulfate solution,* stopper, and store in a dark place for 7 hours.

Color Reading. Determine the percentage of transmission of about 5 ml. in a 10-mm. cell in the Pulfrich photometer, set in vertical position, using an S–57 yellow filter.

Calculation. Compare with a curve plotted on a graph with milligrams of selenium per 100 ml. of tissue digest as abscissas and percentage of transmission as ordinates.

III. Davidson Wet Combustion Modification.[176] Although Schmidt states that a considerable amount of iron interferes with the color formation, Davidson, of the U. S. Bureau of Chemistry and Soils, found that the presence of a certain amount is essential for developing a stable and lasting color.

Apparatus. *Colorimeter,* Schreiner or Klett, or *Nessler Tubes.*

Reagents. *Codeine Sulfate, 2%* solution in water or the alkaloid dissolved with the aid of a few drops of H_2SO_4.

Standard Solutions. Prepare a stock solution by dissolving 1 g. of selenium in HNO_3, evaporating nearly to dryness, and diluting to 1 liter with water. To dilutions of the stock solutions containing from 20 to 200 γ of selenium, add selenium-free plant material similar in kind and amount to the unknown and digest in like manner. If such is not available, add to the standard solution near the close of the digestion period the ash of the sample of the material being analyzed, comparable in weight with that of the unknown, which has been freed from selenium by ashing with KH_2PO_4 at 700°. Usually it is sufficient to add 1 ml. each of *0.5%* $FeSO_4 \cdot 7H_2O$ *solution,* prepared with the addition of a few drops of H_2SO_4, and a molecular solution of *mono- or dipotassium phosphate.*

Process. *Digestion.* To a quantity of the sample, containing not less than 20 γ of se-

lenium and preferably between 50 and 200 γ, add *sulfuric acid* and 0.7 g. of *mercuric oxide* and digest as in the Kjeldahl method. Usually 10 g. of the sample require about 75 ml. and 5 g. about 60 ml. of the acid, leaving 20 to 25 ml. of solution at the end of the digestion. Running over is avoided by using unground material, a large flask, and a system of intermittent heating. When selenium-free plant material is used with the standard, digest with the same quantity of *sulfuric acid* as used in the actual analysis; in the other standard, 25 ml. are sufficient.

Color Formation. Cool the digests, make up with *sulfuric acid* to the same volume (usually 30 ml.), return to the flasks, stopper, and let stand overnight. If the supernatant liquid is clear, pipet aliquots (usually 20 ml.) directly into small Erlenmeyer flasks; if turbid, filter before removing the aliquot. In either case, add slowly with shaking 5 to 6 drops of *2% codeine sulfate solution* or *codeine alkaloid*, stopper, and let stand about 2 hours. The quantity of codeine added must be not less than 30 times the quantity of selenium present, hence if more than 200 γ, add a larger quantity of the *reagent*.

Color Comparison. Make the comparison in the Schreiner, Klett, or other colorimeter, or in ordinary Nessler tubes.

TIN

See also Part II, F2.

Aside from the treatment of molasses with tin salt to lighten the color, long since discontinued, the presence of tin in food is due to contamination from the wrapping or container. Extensive data obtained twenty-five years ago [177] showed that canned corn and peas contained very small amounts of tin, tomatoes more, but, strange to say, certain non-acid vegetables, notably pumpkin and string beans, contained the highest amounts.

In the examination of canned vegetables and fruits, it is desirable to weigh separately the liquor and the drained substance of a can, determine the tin content of each, and from the data calculate the tin content of the total contents.

Baker Sulfide-Aluminum Reduction Iodometric Method.[178] PROCESS. *Incineration and Solution.* (*A*) *Hilger and Leband Procedure.*[179] Weigh the contents of a can, evaporate and ignite in a porcelain dish, and fuse the ash with *sodium hydroxide* in a silver crucible. Remove the melt from the crucible by boiling with several portions of water, add *hydrochloric acid* so as to have 2 ml. in excess of that required to neutralize the alkali, and filter.

(*B*) *Smith and Bartlett Wet Combustion Procedure.*[180] Weigh out a suitable quantity of the sample (50 g. of flesh foods, 100 g. of vegetables or fruits) into a porcelain dish and dry roughly. Measure 75 to 100 ml. of *sulfuric acid* into a large Kjeldahl flask, heat until fumes appear, then add in small portions the dried substance, heating after each addition until frothing ceases. Cool and add cautiously to the charred mass 25 ml. of *nitric acid*. After the reaction with evolution of red fumes abates and the solution cools, add a fresh portion of 25 ml. of *nitric acid* and heat until no more nitric fumes appear and the organic matter forms with the acid a homogeneous solution. Boil 45 minutes, add 10 to 15 g. of *potassium sulfate* when danger of frothing is over, and continue the boiling for 3 to 5 hours or until the solution is colorless. Transfer the solution to a large beaker, dilute to about 600 ml. and heat to boiling.

Nearly all the tin separates as partially hydrated stannic oxide, part of which adheres so closely to the sides of the flask as to prevent removal by washing. Filter, thus separating the stannous oxide from other compounds, and transfer the filter and contents to the flask, adding 20 ml. of *saturated sodium hydroxide solution* and 20 ml. of water. Boil the contents of the flask for several minutes, remove the liquid containing the tin

in the form of sodium stannate to a beaker, and acidify as under *A*.

Sulfide Reduction. Dilute the solution, if necessary, neutralize with *ammonium hydroxide*, make 2% acid with *hydrochloric acid*, and thoroughly saturate with *hydrogen sulfide gas*. Allow to settle, filter on a Gooch crudible with a false bottom, and wash three or four times with water. The presence in the precipitate of calcium, phosphorus, silica, and small amounts of lead and iron does not interfere with the subsequent titration.

Reoxidation. Transfer the precipitate together with the asbestos and the removable bottom to a 300-ml. Erlenmeyer flask and boil with *hydrochloric acid*, adding dry *potassium chlorate* from time to time to insure the complete decomposition and solution of the tin sulfide and the elimination of sulfur.

Aluminum Reduction. Add to the solution in the Erlenmeyer flask a few strips of *tin-free aluminum foil* until all the chlorine is removed, then connect with a Kipp carbon dioxide generator and scrubber by means of a bulb delivery tube passing through a double-bored rubber stopper. Through the second hole of the stopper pass an exit tube that dips through 20 cm. of water in a cylinder forming a water seal and a stabilizer of the gas flow. Raise the tube in the cylinder and pass *carbon dioxide* rapidly into the liquid and, when the air is completely expelled, raise the rubber stopper, drop 1 g. of *aluminum foil* into the flask, and replace the stopper. Boil on a hot plate for a few minutes during the evolution of hydrogen and the reduction of the tin to stannous chloride, then cool in ice water without breaking the carbon dioxide insulation. After the action of the aluminum foil, the acid strength is reduced from 30 to 40% to about 25 to 30%.

Titration. Without removing the asbestos, lift the stopper and run an excess of *standard 0.01 N iodine solution* into the flask while continuing the current of *carbon dioxide*, then titrate back with *standard 0.01 N sodium thiosulfate solution*, using *starch paste* as indicator. For most purposes it is satisfactory to titrate directly with *standard iodine solution*, after washing off the tubes with air-free water, prepared by boiling with a little *sodium bicarbonate*, and acidifying with *hydrochloric acid*.

Glassmann and Barsutskii Dichromate Volumetric Method.[181] As developed at the Odessa Industrial Food Laboratory, the method is briefly as follows.

Reagent. *Standard Potassium Dichromate Solution*, 0.02 *N*. Standardize against pure tin treated as in the analysis.

Process. *Ignition and Solution.* Dry 50 g. of the sample, ignite, and extract the ash with *1 + 2 nitric acid*. Filter to remove iron, copper, and lead and reignite the paper and residue.

Cyanide Reduction. Reduce the tin to the metallic form by fusion at a low red heat with 1 g. of *potassium cyanide*. Extract the mass with 150 ml. of water and filter, then digest the residue and paper with 25 ml. of *hydrochloric acid* in a flask fitted with a Bunsen valve. To complete the reduction, add 1 g. of *zinc* and cool in *carbon dioxide*.

Titration. Using *potassium iodide* and *starch solution* as indicators, titrate with 0.02 *N potassium dichromate solution*.

Examples. Canned fish 17.5 to 154.6 γ/g. of tin. A sample of canned *Gobius* stored 32 months contained 154.6 and on standing 12 days in contact with the can after opening reached 420 γ/g. of tin.

Höltje Iron Reduction Iodometric Method.[182] This method is characterized by its simplicity.

Apparatus. The bulb flask with its long neck is about 15 cm. long. Into the neck extends the delivery tube for carbon dioxide, drawn out at the end to a narrow diameter.

Process. *Reduction.* Transfer the slightly acid solution (5 to 10 ml.), containing the tin obtained by wet or dry combustion (see above), into the flask and add 10 to 20 mg. of

potassium iodate and 0.1 g. of *iron powder.* Heat while passing a slow stream of *carbon dioxide* into the flask and boil for 10 minutes. Remove the heat, add 1 ml. of *air-free 24% hydrochloric acid,* and boil briskly for 20 minutes, thus dissolving most of the iron powder. With the flask upright, raise the tube, add 0.1 g. of *iron powder* through a dry funnel, wash down with air-free water, replace the tube, and boil briskly for 15 minutes. Add 0.05 g. of *iron powder* and again boil for 15 minutes. Add a fresh portion of 0.05 g. of *iron powder* and 0.5 ml. of the *hydrochloric acid,* then boil for 30 minutes, after which only a small amount of iron remains undissolved. In order to bring about a complete solution of the iron powder, add a final portion of 1 ml. of the *hydrochloric acid* and boil 10 to 15 minutes.

Titration. Cool the solution which now measures about 10 ml. While still running the *carbon dioxide* into the flask, add a drop of *1% starch paste* and dropwise sufficient *standard iodine solution* just to produce a blue color, then titrate back with *standard thiosulfate solution.*

Zinc

The presence of zinc in foods was long overlooked. Contamination from machinery and containers began with the invention of the galvanizing process. Fruit dried on galvanized trays may contain 0.01% or more, rarely as high as 0.10%. When zinc chloride is used as a flux in soldering cans, a small amount may remain in the seams and, if not removed by washing, contaminate the contents.

Many, perhaps most, of the results in the literature are erroneous owing to zinc present in the reagents or extracted from Jena or other glass ware. Only Pyrex glass ware should be used.

Cereals appear to contain 25 to 50 γ/g., the flour less, the bran and germ more (as high as 175). Leguminous seeds and leaf vegetables contain less than the lowest limits for cereals, all calculated to the dry basis. In milk, whether or not from containers, 0.02 to 0.05, in lean meat 15 to 50, and in liver as high as 100 to 200 γ/g., fresh basis, have been reported.

Mylius Ferrocyanide Nephelometric Method. This method, first proposed by Mylius,[183] has been variously modified in recent years with the view of increasing its accuracy and convenience.

I. Breyer-Birckner-Bodansky Modification.[184] Reagents. *Standard Zinc Chloride Solution.* Dissolve 1.2447 g. of ignited ZnO in HCl, sufficient to leave the solution clear and faintly acid, and dilute to 1 liter. For use, dilute 10 ml. to 100 ml.; 1 ml. = 0.1 mg. of zinc.

Potassium Ferrocyanide Solution. Dissolve 34.8 g. of $K_4Fe(CN)_6 \cdot 3H_2O$ in water and dilute to 1 liter. Keep in the dark.

Process. *Incineration and Solution.* Moisten the material with *sulfuric acid* and heat, adding *nitric acid* to aid in the oxidation. Reduce to ash at a dull red heat. Extract the ash repeatedly with hot *dilute hydrochloric acid,* filter the combined extracts, and evaporate to dryness. Take up the residue with 2 ml. of *hydrochloric acid* and 50 ml. of water.

Sulfide Precipitation. Transfer the solution to a flask, precipitate the copper with *hydrogen sulfide gas* and filter. Boil the filtrate until the hydrogen sulfide has been driven off, cool, and neutralize with *ammonium hydroxide.*

Calcium Citrate Precipitation. Add 10 ml. of *50% citric acid solution* and heat to boiling. If there is no separation of calcium citrate, add small portions of *calcium carbonate* until about 1 g. of calcium citrate precipitates. While still hot, run *hydrogen sulfide gas* rapidly into the liquid, continuing until the liquid is cold. Allow to stand for several hours with occasional heating on the water bath until the supernatant liquid is clear.

Filter and wash the zinc sulfide with *2% ammonium thiocyanate solution.*

Resolution. Dissolve the precipitate on the paper with 3 ml. of hot *hydrochloric acid* diluted somewhat with water, collecting the filtrate in the flask previously used for the precipitation. If a reddish color, due to ferric thiocyanate, appears, the zinc should be reprecipitated; a turbidity, due to sulfur, however, may be removed by boiling.

Nephelometric Comparison. It is essential that the liquid be clear and colorless. Dilute the entire solution of the zinc sulfide or a suitable aliquot to 45 ml. in a 50-ml. Nessler tube and add 3 ml. of hydrochloric acid. Prepare in other similar tubes a series of solutions containing various measured amounts of a *standard zinc chloride solution,* 3 ml. of *hydrochloric acid,* and distilled water up to 45 ml. Pipet into each tube 5 ml. of *potassium ferrocyanide solution,* mix quickly, and compare the turbidity of the unknown with that of the standards, looking down through the columns held over a sheet of fine print. A known solution nearly matching the unknown may be adjusted to an exact match by further addition of the standard solution.

II. Fairhall and Richardson Modification.[185] These authors do not remove the copper from the solution of the ash by separate precipitation with hydrogen sulfide, nor do they employ calcium carbonate to form with citric acid a small amount of insoluble calcium citrate, as an aid in securing a clear filtrate, but depend on the coprecipitation of copper and zinc sulfides to perform this function. The sodium citrate which they use appears to serve merely to hold the iron in solution. They consider that the nephelometric ferrocyanide method gives accurate results only within definite limits of acidity and salt concentration. It has been successfully employed by the originators, at the Harvard School of Public Health, in the analysis of blood and other biological materials.

Apparatus. *Comparison Tubes.* If Nessler tubes are used, blacken the bottom and a band about the graduation.

Process. *Incineration.* Weigh out a quantity representing about 10 to 20 g. of dry matter, incinerate as recommended by Thompson[186] at 450° (low redness), and dissolve the ash in *6 N redistilled hydrochloric acid* and hot water. Dilute to 75 ml., add 5 g. of pure *sodium citrate,* 2 mg. of *copper* (8 mg. of $CuSO_4 + H_2O$), and *thymol blue indicator;* then add *dilute potassium hydroxide solution* until the color is yellow, followed by *bromochlorophenol blue.* If the solution is bluish, add *dilute hydrochloric acid* until the yellow color is just restored.

Sulfide Precipitation. Saturate with *hydrogen sulfide gas* in the cold, filter, and wash the joint sulfides of zinc and copper until practically all the iron has been removed. Dissolve the sulfides in *hydrochloric acid* and *nitric acid* in a silica or porcelain dish and evaporate twice to dryness with *hydrochloric acid.* Dissolve the residue in *hydrochloric acid* and repeat the precipitation with *hydrogen sulfide gas,* omitting the *sodium citrate,* but taking special care in the adjustment of the acidity.

Resolution. Dissolve the reprecipitated zinc and copper sulfides as before, add 5 ml. of *6 N hydrochloric acid* diluted with 20 ml. of water, then slowly saturate the cold solution with *hydrogen sulfide gas.* and filter. Evaporate to dryness the filtrate containing the zinc chloride, moisten thoroughly the residue with 4 to 5 drops of *6 N hydrochloric acid,* and warm slightly to insure complete solution; then add a little water. Transfer to a 25-ml. volumetric flask and make up to the mark.

Nephelometric Comparison. To a 5- or 10-ml. aliquot of the 25-ml. solution of the zinc chloride, add 10 ml. of *0.1341 N potassium hydroxide solution,* exactly neutralize with *0.10 N hydrochloric acid,* then add 1 ml. of the *acid* in excess, and dilute almost to 50 ml. in

a comparison tube or jar. Add 1 ml. of *2% potassium ferrocyanide solution,* fill to the 50-ml. mark, and mix. The solution so prepared is 0.002 N in acid content and has a concentration of 0.0268 M with respect to potassium chloride.

Make opacity comparisons against *standard zinc solutions* having the same acid and salt concentrations as the solution of the unknown and a range from 0.05 to 0.50 mg. in steps of 0.05 mg. of zinc in the 50 ml., 0.20 to 0.25 mg. being the most satisfactory amount.

Fischer Dithizone Colorimetric Method. This method is based on the color reactions of dithizone (diphenyl-thiocarbazone) with heavy metals, as brought out by Fischer (Berlin) publishing alone [187] and with Leopoldi (see Lead).[188]

I. Fischer and Leopoldi Modification. The procedure closely resembles that for lead, but differs in that the excess of dithizone is removed by hydrogen sulfide or ammonium sulfide and interfering metals are buffered or removed by special treatment. Fischer and Leopoldi's paper is much involved and the instructions are in parts indefinite.

APPARATUS. *Colorimeter.*

REAGENTS. Shake all reagents with dithizone solution in carbon tetrachloride until free from metals giving color reactions with that reagent.

Dithizone Solution. Dissolve 10 mg. of diphenylthiocarbazone in 100 ml. of CCl_4.

Acid Salt Mixture. Make up to 500 ml. 300 ml. of 5% sodium acetate solution, 10 ml. of 50% $Na_2S_2O_3 \cdot 5H_2O$ solution, and 40 ml. of 10% HNO_3.

PROCESS. *Precipitation.* After ashing or digesting the material, prepare a dilute acid solution containing 5 to 40 γ of zinc and evaporate nearly to dryness to remove the excess of acid. Dilute to 10 to 20 ml., add 1 to 2 ml. of *10% sodium hypophosphite solution,* and boil for a few minutes with stirring to aid the flocculation of the metallic precipitate. Filter on an ash-free paper and wash.

Dithizone Extraction. Place the paper with precipitate in a separatory funnel and extract with successive portions of *dithizone solution in carbon tetrachloride* until the last portion remains pure green.

Buffer Treatment. Add to the dithizone extract with shaking *5% sodium acetate solution* until the color of *Congo paper* changes to red, then enough to equal in volume half that of the liquid. Finally add 3 ml. of *5% sodium thiosulfate solution* for each 10 ml. of liquid.

If the color of the tetrachloride phase is green or greenish, zinc is not present. If, however, it is yellow-brown or a dirty mixed color (Ag, Cu, Hg, Au, Bi) or red or violet changing to green with *hydrogen sulfide* (Pb, Cd, Cu), the treatment should be repeated on a new solution, more thiosulfate being used.

A red tetrachloride phase, unchanged by *hydrogen sulfide* but changing to green with *ammonium sulfide solution,* is a positive test for zinc.

Treatment with Hydrocyanic Acid. A red or violet tetrachloride phase, becoming green with the separation of dark flakes with either *hydrogen sulfide* or *ammonium sulfide solution,* indicates lead, thus necessitating a new solution with addition of a larger amount of thiosulfate and treatment with sodium cyanide in faintly acid solution.

Remove the foreign metals from the dithizone solution by shaking two or three times with 5 ml. of an *acid salt mixture,* then wash once with water and twice with *10% sodium sulfide solution* to remove the excess of dithizone.

Color Comparison. Draw off the dithizone from the aqueous solution and make the color comparison with suitable standards treated in the same manner as the unknown.

II. Hibbard Modification.[189] Of two procedures, given by Hibbard (University of Southern California) for the removal of copper and lead respectively, only the former, applicable to natural products containing no

appreciable amounts of lead, is here described. Spray residues rich in lead contain little or no zinc.

Apparatus. All utensils must be zinc-free. Pyrex is satisfactory; Jena glass is not.

Reagents. *Water*, zinc-free. Distil in Pyrex apparatus.

Hydrochloric Acid, zinc-free. If the c.p. HCl is not satisfactory, run into H_2SO_4 and absorb the HCl gas in pure water. Distillation is useless since the zinc passes over.

Ammonium Citrate Solution, zinc-free. Add to 10% $C_6H_8O_7 \cdot H_2O$ solution NH_4OH to alkaline reaction and shake with dithizone and $CHCl_3$ until no more zinc is extracted.

Dithizone Solution. Dissolve 15 mg. in 100 ml. of $CHCl_3$.

Chloroform. It should dissolve dithizone easily to a dark green solution which, greatly diluted, is still green, not changing to yellow. Recover by distillation with a small amount of $Na_2S_2O_3 \cdot 5H_2O$ and a very small amount of NaOH.

Standard Zinc Solution. Prepare a stock solution by dissolving 1 gram of c.p. zinc in H_2SO_4 and diluting to 1 liter; 1 ml. = 1 mg. of zinc.

Process. *Dithizone Extraction*. To the acid solution of the sample in a separatory funnel, add *dilute ammonium hydroxide* to alkaline reaction. If a precipitate forms, add *hydrochloric acid* until dissolved, then 1 to 2 ml. of *ammonium citrate solution*, followed by *ammonium hydroxide* to alkaline reaction. To the clear liquor, add several drops of *dithizone solution* and 5 ml. of *chloroform*, then shake for a few seconds. On settling, the chloroform layer will be green and the aqueous layer brown-yellow, if zinc or other heavy metal is absent, or some shade of red if zinc is present. Add more *dithizone solution*, shake, and repeat until the chloroform becomes bluish or purplish from excess of dithizone or a modified shade if other metals are present. Draw off the chloroform layer and shake out the watery mixture with a fresh portion of the *dithizone solution* and *chloroform*, after which the chloroform solution should be green or slightly reddish if practically all the zinc has been removed.

Ammonia Treatment. To the combined dithizone extracts, contained in a second separatory funnel, add three times the volume of *0.02 N ammonium hydroxide*, shake, and siphon off the aqueous layer, repeating the treatment until all the dithizone is removed and the chloroform is reddish and the aqueous solution colorless.

Acid Treatment. Shake the dithizone-chloroform solution with 5 ml. of *0.5 N hydrochloric acid*, and draw off the chloroform solution. Wash the acid solution with a little *chloroform* and add this latter to the other chloroform solution. Shake the combined chloroform solutions with a fresh portion of 5 ml. of *0.5 N acid* and draw off the chloroform which contains the copper.

Ammonia Treatment. To the combined acid solutions, which contain the zinc, add *ammonium hydroxide* to alkaline reaction, shake out with *dithizone solution*, and remove the excess of the reagent as before.

Color Comparison. Compare the chloroform solution of the unknown with one or more *standard solutions* prepared from a stock solution, treated in like manner.

III. Holland and Ritchie Modification.[190] At the Massachusetts Agricultural Experiment Station, sodium diethyldithio-carbamate (see Carbamate Method under Copper) is used in conjunction with dithizone.

Apparatus. *Duboscq Colorimeter*, or its equivalent.

Reagents. Prepare new lots of reagents often and store in zinc-free glass. Distil all water from glass.

Hydrochloric Acid, 0.2 *N*. Distil the acid into cold, metal-free water by allowing it to drip from a separatory funnel below the surface of hot H_2SO_4 and dilute to 0.2 *N*.

Ammonium Hydroxide, 0.2 *N*. Redistil into metal-free water and dilute to 0.2 *N*.

Carbamate Reagent. Dissolve 2.5 g. of sodium diethyldithio-carbamate in water and dilute to 1 liter.

Dithizone Reagent. Dissolve 0.015 g. of crushed dithizone (diphenyl-dithiocarbazone) in 10 ml. of the 0.2 N NH_4OH and transfer to a 250-ml. separatory funnel with 90 ml. of water. Shake out with 10-ml. portions of CCl_4 to a green color. Discard the solvent layers and filter the aqueous layer through washed ashless paper. Prepare daily.

Citrate Buffer. Dissolve 225 g. of $(NH_4)_2HC_6H_5O_7$ in water, add 0.2 N NH_4OH until distinctly alkaline to litmus, and dilute to 2 liters. Transfer 250 ml. to a 750-ml. separatory funnel, add an excess of dithizone reagent (usually 3, 2, and 1 ml. respectively), and shake out with three 25-ml. portions of CCl_4 to a green color. Discard the solvent layers and filter the aqueous layers through washed ashless paper.

Standard Zinc Solution. Boil 1 g. of dry reagent granular zinc with 100 ml. of water and an excess of HCl until the zinc dissolves. Cool and dilute to 1 liter in a volumetric flask; 1 ml. = 1 mg. of zinc. Pipet 2 ml. of this stock solution into a 1-liter volumetric flask and dilute to the mark at 25°; 5 ml. = 10 γ of zinc.

Process. *Incineration.* Reduce 4 g. of the sample, ground to pass a 1-mm. mesh, in an electric furnace below redness, to a white or gray ash. Pulverize and reheat if necessary. Transfer to a 100-ml. volumetric flask with small portions of water, add *hydrochloric acid* dropwise until faintly acid to *litmus*, then add 20 ml. of *0.2 N hydrochloric acid* in excess. Heat on a steam bath, cool, make up to volume, and filter through dry ashless paper.

Dithizone-Tetrachloride Separation. Pipet 10 ml. of the solution (= 0.4 g. of the sample) into a 250-ml. separatory funnel, add 20 ml. of water, 7 ml. of *0.2 N ammonium hydroxide*, 10 ml. of *citrate buffer*, and *dithizone reagent* to a yellow color. Add 10 ml. of *carbon tetrachloride* and extract copper, lead, and zinc (also cobalt, cadmium, and mercury if present) by shaking vigorously for at least 2 minutes. Allow to separate, draw off the solvent layer into another separatory funnel, and discard the yellowish ammoniacal aqueous portion containing non-reacting acids and bases. To the solvent layer add 45 ml. of water and 5 ml. of *0.2 N hydrochloric acid* and isolate the copper by shaking. The lead and zinc remain in the colorless aqueous solution. To the acid layer, add 19 ml. of water, 15 ml. of *0.2 N ammonium hydroxide*, 10 ml. of the *citrate buffer*, 5 ml. of *carbamate reagent*, and *dithizone reagent* dropwise to a yellow tint. Finally add 10 ml. of *carbon tetrachloride* and extract the colored zinc salt by shaking. Allow to separate, rinse the delivery tube with a few drops of the solvent layer, draw off the remainder through a dry ashless paper (to remove moisture) into a stoppered weighing bottle.

Color Comparison. Make the comparison against 5 ml. (= 0.01 mg.) of the dilute *standard zinc solution* treated in like manner as the unknown in a Duboscq colorimeter (or its equivalent), using the micro cup and a green color filter.

Blank. Make a blank determination on the reagents and deduct the reading from that of the unknown.

Calculation. Obtain the gammas per gram (γ/g.) of zinc (M) in the sample by the formula

$$M = \frac{0.002 \times 5 \times 1000 \times R}{R_1}$$

in which R and R_1 are the scale readings of the standard and the unknown respectively, and 0.002 is the milligrams of zinc in the volume of standard zinc solution used, and 0.4 is the grams of the sample in the aliquot.

IV. Cowling and Miller Modification.[191] The following procedure for the determination of small amounts of zinc originated at the Michigan Agricultural Experiment Station.

APPARATUS. *Photoelectric Colorimeter*, with Sextant green (Corning No. 401) filter.

REAGENTS. Purify and store all reagents in Pyrex glass containers.

Ammonium Hydroxide Solution, 1.0 *N*. Distil into water in Pyrex apparatus one-half volume of NH_4OH and dilute to 1.0 *N*.

Standard Hydrochloric Acid, 1.0 *N* and 0.02 *N*. See Hibbard Modification above.

Dithizone Reagent. Dissolve 0.2 g. of diphenyl-thiocarbazone in 500 ml. of CCl_4 and filter. Shake the filtrate with 2 liters of 0.02 *N* NH_4OH (40 + 1960) and discard the tetrachloride phase. Repeat the treatment of the aqueous phase, using, however, 100 ml. of CCl_4, until the latter is pure green, then add 500 ml. of CCl_4 and 45 ml. of 1.0 *N* HCl to the aqueous solution, shake, separate, and dilute the CCl_4 solution to 2 liters. Store in a brown bottle in a dark place.

Ammonium Citrate Solution, 0.5 *M*. Dissolve 226 g. of $(NH_4)_2HC_6H_5O_7$ in 2 liters of water, add NH_4OH (80 to 85 ml.) to *p*H 8.5 to 8.7, then add an excess of dithizone reagent—an orange-yellow coloration should form in the aqueous phase after shaking—and extract with 100-ml. portions of CCl_4 until the latter takes on a full green coloration. Separate the aqueous phase and store in Pyrex.

Carbamate Solution. Dissolve 0.25 g. of sodium diethyldithio-carbamate in water and dilute to 100 ml. Prepare fresh as needed.

Citrate Solution A. Dilute 1 liter of 0.5 *M* $(NH_4)_2HC_6H_5O_7$ solution and 140 ml. of 1.0 *N* NH_4OH to 4 liters.

Citrate Solution B. Dilute 1 liter of 0.5 *M* $(NH_4)_2HC_6H_5O_7$ solution and 300 ml. of 1.0 *N* NH_4OH to 4.5 liters. Just before use, add 9 volumes of this mixture to 1 volume of freshly prepared carbamate solution.

Standard Zinc Solution. Weigh 0.25 g. of pure zinc into a 250-ml. volumetric flask, heat with 50 ml. of water and 1 ml. of H_2SO_4 until dissolved, then make up to volume.

PROCESS. *Incineration*. Burn to an ash 5 g. of the finely ground air-dry sample in a platinum dish, heated in an electric muffle at 500 to 550°. Add a little water, then 10 ml. of *1.0 N hydrochloric acid* and heat on a boiling water bath. Dilute with 5 or 10 ml. of hot water, filter on a 7-cm. Whatman No. 42 paper (previously washed with two 5-ml. portions of hot 1.0 *N* hydrochloric acid, then with hot water) and collect the filtrate in a 100-ml. volumetric flask. Wash the paper with hot water, add a drop of *methyl red indicator* to the filtrate, then *1.0 N ammonium hydroxide* until neutral, followed by 4 ml. of *1.0 N hydrochloric acid*. Cool and dilute to volume.

Dithizone Complex Extraction. Pipet a 10-ml. aliquot of the ash solution, containing not more than 30 γ of zinc, into a 125-ml. separatory funnel, add 40 ml. of *citrate solution A* and 10 ml. of *dithizone reagent*, shaking vigorously for 30 seconds. (If a volume other than 10 ml. of ash solution is used, add 1 ml. of *0.2 N hydrochloric acid* for each 5 ml. less, or 1 ml. of *0.2 N ammonium hydroxide* for each 5 ml. more than 10 ml. If the aqueous phase is not colored red or yellow, add more dithizone.) Draw off the carbon tetrachloride extract into a second funnel and rinse down with 1 or 2 ml. of carbon tetrachloride any carbon tetrachloride extract, repeating if necessary. Add 5 ml. of *carbon tetrachloride* to the aqueous liquid in the first funnel, shake for 30 seconds, allow to separate, and run off the carbon tetrachloride layer into the second funnel, rinsing with 1 or 2 ml. of *carbon tetrachloride* as before. If the carbon tetrachloride is not a clear green, repeat the extraction and rinsing.

Copper Removal. Add to the carbon tetrachloride extract in the second funnel 50 ml. of *0.02 N hydrochloric acid*, shake vigorously for 1.5 minutes, allow to settle, draw off the carbon tetrachloride layer, and rinse as in the first extraction until all traces of green dithizone are removed. Remove the stopper and

allow the carbon tetrachloride on the surface of the aqueous layer to evaporate.

Zinc Extraction. Pipet 50 ml. of *citrate solution B* and 10 ml. of *dithizone reagent* into the 50 ml. of *0.02 N hydrochloric acid solution.* Shake for 1 minute, allow to separate, flush out the stopcock and stem with 1 ml. or so of the carbon tetrachloride extract, and collect the remainder in a test tube.

Color Measurement. Pipet 5 ml. of the carbon tetrachloride extract into a 25-ml. volumetric flask, dilute to volume with *carbon tetrachloride,* and measure the light transmission within 2 hours in a photoelectric colorimeter provided with a Sextant green (Corning No. 401) filter, protecting the solution from light as much as possible.

CALCULATION. Compare with a standard curve.

Todd and Elvehjem Phosphomolybdate Colorimetric Method.[192] The steps are (1) ashing and extraction, (2) coprecipitation of zinc and copper as sulfide in the presence of sodium citrate, (3) later removal of copper sulfide by precipitation in a strongly acid solution, (4) precipitation of zinc ammonium phosphate, (5) precipitation of the phosphoric acid of the last as ammonium phosphomolybdate, and (6) determination of phosphoric acid colorimetrically by the Fiske and Subbarow method, employing reduction of the phosphomolybdate precipitate with aminonaphthol-sulfonic acid. The last two steps are carried out in the same solution. Although the procedure may seem complicated, each step is well defined and not laborious.

APPARATUS. *Colorimeter.*

REAGENTS. *Sulfuretted Wash Liquid.* Hydrogen sulfide water buffered with sodium citrate solution, adjusted to pH 3.5.

Molybdate Solution:

(*A*) 2.5% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ solution in 5 *N* H_2SO_4;

(*B*) 2.5% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ solution in 3 *N* H_2SO_4.

PROCESS. *Incineration and Solution.* Burn to a carbon-free ash a quantity of the sample containing about 0.5 mg. of zinc. Take up in *1 + 1 hydrochloric acid,* heat, filter, and wash up to about 30 ml. in a 100-ml. Erlenmeyer flask.

Sulfide Precipitation. Add to the solution 5 ml. of *1 + 3 sodium citrate solution,* 2 mg. of *copper* in the form of copper sulfate solution, and *saturated potassium hydroxide solution* to pH 3.5, as shown by a few drops of *bromophenol blue indicator.* Heat to boiling, run in a stream of *hydrogen sulfide gas,* continuing the current while cooling. Transfer to a 50-ml. Pyrex centrifuge tube, rinsing the flask once with 10 ml. of *sulfuretted wash liquor.* Centrifuge at 1500 r.p.m. for about 5 minutes, decant, and reject the supernatant liquid. Wash the precipitate twice with 15-ml. portions of the *sulfuretted wash liquid,* centrifuging after each addition and discarding the liquid. Break up the sulfide precipitate with a fine stream of water (about 3 ml.), add 0.5 ml. of *hydrochloric acid,* and heat in a boiling water bath. Add dropwise 0.5 ml. of *30% hydrogen peroxide solution,* heating if oxidation is not complete in a few minutes.

Transfer the solution to the original Erlenmeyer flask and evaporate over a low flame to small volume. Dilute to 30 ml., add 5 ml. of *1 + 3 sodium citrate solution,* adjust to pH 3.5, precipitate with *hydrogen sulfide gas,* and filter as before. Oxidize the precipitate with *30% hydrogen peroxide solution in hydrochloric acid,* and evaporate nearly to dryness.

Take up in 22 ml. of water and 2.5 ml. of *1 + 1 hydrochloric acid,* run in *hydrogen sulfide gas* for 5 minutes, transfer to a 50-ml. centrifuge tube, and rinse the flask twice with 5-ml. portions of *sulfuretted wash liquid.* Centrifuge 5 minutes, decant into the original Erlenmeyer flask, wash the precipitate with 15 ml. of the *wash liquid* used for the rinsing, centrifuge 5 minutes, add the clear liquid to that in the Erlenmeyer flask, and evaporate nearly to dryness. Transfer to a 15-ml. grad-

uated centrifuge tube with water, keeping the volume below 5 ml.

Phosphate Precipitation. Adjust the *p*H to 6.6 with *ammonium hydroxide*, using *bromothymol blue* as indicator. Heat in a hot water bath to 90°, add dropwise 0.5 ml. of *10% diammonium phosphate solution*, and allow to stand at 90° for 30 minutes. Cork, allow to stand 4 hours, centrifuge for 5 minutes, decant off the supernatant liquid, and wash with 5 ml. of *1% diammonium phosphate solution*. Wash the precipitate further with 10 ml. of *50% ethanol*, whirl, discard the ethanol, and allow the tube to drain in an inverted position.

Molybdate Precipitation, Reduction, and Color Comparison by the Fiske and Subbarow Method.[193] Dissolve the zinc ammonium phosphate precipitate in 8 ml. of *2 N sulfuric acid*, transfer to a 50-ml. centrifuge tube, add 5 ml. of *molybdate solution B* and 2 ml. of *aminonaphthol-sulfonic acid*, dilute to volume, and compare the color with that of a *standard solution* containing 2 ml. of *stock standard phosphate solution* and 5 ml. of *molybdate solution A*.

Calculation. Calculate the zinc from the phosphorus obtained and the ratio of zinc to phosphorus in the formula $ZnNH_4PO_4$.

Lang-Hibbard Iodometric Method. Credit for the process of separation of the zinc from interfering substances is due to Hibbard,[194] of the University of California, and for the iodometric method of titration to Lang.[195]

Reagents. *Citrate Buffer.* Dissolve 12 g. of $Na_3C_6H_5O_7 \cdot 2H_2O$ and 23 g. of $H_3C_6H_5O_7 \cdot H_2O$ in water and make up to 100 ml. Adjust to *p*H 3 by adding one or the other constituent as required.

Talc Suspension. Wash powdered talc with acid and prepare a 5% suspension.

Sulfide Wash Liquid. Saturate water with H_2S gas and add 5 ml. of 90% formic acid per liter.[196]

Phosphate Buffer. Mix 25 g. of KH_2PO_4 with 5 ml. of sirupy H_3PO_4 and dilute to 100 ml. Adjust to *p*H 3 by adding H_3PO_4 or KOH. For use, make up to 1 liter.

Standard Sodium Thiosulfate Solution, 0.01 *N*. Prepare this stock solution in cold boiled water for dilution each day to 0.001 *N*.

Process. *Separation from Other Metals as Sulfide. A. Traces of Arsenic and Lead Present* (Less than 2 mg. of arsenic and 10 mg. of lead in the sulfide precipitate). Incinerate 5 g. of the ground air-dry material in a porcelain dish at low red heat, avoiding fusion. To the ash, add 15 ml. of water and 7 ml. of *3 N hydrochloric acid* and evaporate on the steam bath to about half volume. Filter into a small flask (50 to 100 ml.) and wash with hot water to a volume of about 25 ml. Filtration is unnecessary if the insoluble matter is slight and further incineration is required only when the unburned carbon is considerable.

Add a few drops of *bromophenol blue indicator*, then *ammonium hydroxide* until the solution is blue, followed by sufficient *1.0 N hydrochloric acid* to cause the appearance of a yellow color and complete solution. To hold the iron in solution and maintain the proper *p*H, add 3 to 5 ml. of *citrate buffer*, according to the amount of iron present. Heat on the steam bath, then add *dilute acid* or *ammonium hydroxide* until the color is gray (neither yellow nor blue) with a *p*H of 3 to 3.4. In the presence of ferric iron, sufficient to interfere with the adjustment of the *p*H with the indicator, previous reduction by *sulfur dioxide* from the compressed gas or sodium sulfite is desirable.

Run into the hot liquid a rapid stream of *hydrogen sulfide gas* for 1 or 2 minutes. When neither lead nor copper is present in sufficient amount to form with the zinc a considerable precipitate of sulfides, add 1 ml. of *acid-washed powdered talc suspension* (about 0.05 g.) to aid in the filtration. Place the flask in cold water and continue to run *hydrogen sulfide gas* into the liquid until cold. ...o stand overnight or until

the precipitate settles. Filter and wash on a 7-cm. close-textured (Whatman No. 1) paper five or six times with *sulfide wash liquid.*

B. Larger Amounts of Arsenic and Lead Present. Add *hydrochloric acid* to the solution of the ash in the small flask sufficient to make it 6 *N*, run in *hydrogen sulfide gas* to saturation, cork the flask, and heat on a steam bath. Collect the coagulated yellow arsenic sulfide on a filter and wash. Evaporate the filtrate in a porcelain dish until nearly dry and wash back again into the small flask. Adjust to 0.25 *N* and again run in *hydrogen sulfide gas* until saturated. Cork, heat on a steam bath, and, when the flocculent lead sulfide becomes granular, filter and wash. Return the filtrate again to the small flask, neutralize to *p*H 3, add *citrate buffer*, and precipitate the zinc with *hydrogen sulfide gas* as described under *A*.

Iodometric Titration. Dissolve the zinc sulfide from the filter paper with *1.0 N hydrochloric acid* into a 50-ml. porcelain dish and evaporate to dryness on a steam bath. To the residue, add 0.5 ml. of *dilute phosphate buffer*, 1 to 2 drops of *20% potassium iodide solution*, 3 drops of *0.5% starch paste*, and 3 drops of freshly prepared *1% potassium ferricyanide solution.* The presence of zinc is indicated by the blue color. Titrate rapidly, while gently rolling the casserole to avoid oxidation, with *standard 0.001 N sodium thiosulfate solution* until the blue color changes to pure yellow. A slight blue color, discharged by a drop or two of the thiosulfate solution, is ignored since about 1 ml. is required for 0.1 mg. of zinc. Check the result by titrating a solution containing a known amount of zinc.

The reaction, according to Hibbard, appears to be in two stages:

$$\text{I. } K_3Fe(CN)_6 + KI \rightleftharpoons K_4Fe(CN)_6 + \tfrac{1}{2} I_2$$

$$\text{II. } Zn^{++} + K_4Fe(CN)_6 =$$

Vanselow and Laurance Spectrographic Micro Method.[197] The method was devised at the Citrus Experiment Station of the University of California with the view of extending the spectrographic determination beyond the limits permitted by the Rogers direct arcing method.

APPARATUS. *Concave Grating Spectrograph* (Applied Research Laboratories, Los Angeles). The grating (curvature radius 150 cm.) is ruled for 5.1 cm. with lines 2.54 cm. long. Since there are 9200 lines per cm., the resolving power is several times that of a large quartz prism spectrograph; furthermore the reflecting power is increased by the ruling on speculum metal and aluminized in vacuum. The arc is entirely enclosed, its image being focussed on the grating by a quartz lens. The ultra-violet region from 2370 to 4600 Å is photographed on a film 32 cm. long with the slit in one position. A second slit position serves for photographing the visible spectrum (4580 to 6810 Å), the second order ultra-violet rays being absorbed on a disk of Crookes No. 1 glass.

Calibration. Employ the cadmium internal standard method. Prepare mixtures of known ratios of cadmium sulfide and zinc sulfide, place in the cavity of a graphite electrode 2.5 mg. each of these dry powders and arc as the lower and positive electrode with a direct current of 7 amperes and 150 volts. Although 20 seconds are sufficient to vaporize the sulfides completely, make all exposures for 1 minute, using a slit width of 0.05 mm. and revolving the sector about 500 r.p.m. Compare the zinc line at 3345.0 Å with the cadmium line at 3252.5 Å and plot the ratio LCd/LZn against the square root of the number of milligrams of zinc present for each 2 mg. of cadmium, in which L is the distance between the points at which a spectrum line fades out. The expression $L_1/L_2 = I_2/I_1$ shows the relation of the length to the intensity of two such lines.

Graphite Electrodes, 0.64 cm. in diameter

with a crater 0.32 cm. in both diameter and depth.

REAGENTS. *Acid Citrate Buffer.* Dissolve 120 g. of sodium citrate ($2Na_3C_6H_5O_7 \cdot 11H_2O$) and 230 g. of citric acid ($H_3C_6H_5O_7 \cdot H_2O$) in water, dilute to 1 liter, and adjust to

Formic Acid-Hydrogen Sulfide Reagent. Saturate 0.05 *N* HCOOH solution with H_2S.

PROCESS. *Separation of Zinc.* Thoroughly ash 2 to 4 g. of the dried sample in a sillimanite combustion boat (100 x 20 mm.) at 450° in an electrically heated Pyrex tube combustion furnace. Transfer the ash to a small evaporating dish, add 20 ml. of *1.0 N hydrochloric acid,* then heat on a hot plate until half the solution has evaporated. Filter and wash, keeping the volume within 25 ml. Add to the filtrate, contained in a 125-ml. Pyrex Erlenmeyer flask, 10 ml. of *0.02% cadmium sulfate solution* and precipitate the cadmium sulfides by the following modification of the Fales and Ware method.[198] With *bromophenol blue* as indicator, adjust the solution to about *p*H 3.6 with *saturated potassium hydroxide solution* and add 0.5 ml. of *50% formic acid solution* (sp.gr. 1.2) and 3 to 5 ml. of *acid citrate buffer solution.* Keep the final volume of the solution at about 40 ml. and the *p*H at 2.8 to 3.0. Heat to 60 to 70° and close with a cork stopper provided with hydrogen sulfide inlet and outlet tubes, the former extending nearly to the surface of the solution. Pass *hydrogen sulfide* slowly through the flask during heating. When the boiling point is reached, remove the heat and close the outlet tube. Allow to cool to room temperature without disconnecting the hydrogen sulfide, shaking from time to time. Disconnect, stopper, and allow to stand 30 minutes. Transfer to a 50-ml. Pyrex centrifuge tube, rinsing the flask once with a few milliliters of *formic acid-hydrogen sulfide reagent,* and centrifuge. Pour off the supernatant liquid and wash the precipitate once with 10 to 15 ml. of the *formic acid-hydrogen sulfide reagent* by centrifuging as before. Dry the precipitate overnight in a vacuum calcium chloride desiccator. Transfer the dry precipitate to the crater of the electrode by means of a platinum spatula.

Spectrographic Procedure. See Calibration above.

Reed and Cummings Dropping Electrode Polarographic Method.[199] As developed at Cornell University, the method is designed to correct the defects of the Stout, Levy, and Williams method.[200]

APPARATUS. *Dropping Mercury Electrode,* without rubber connections. See Kolthoff and Lingane.[201] By connection with a manometer, the reproduction of a constant temperature on the mercury column in the electrode and consequently of the dropping rate is permitted.

Heyrovsky Polarograph.

REAGENT. *Ammonium Acetate Solution,* 0.1 *N* of *p*H 4.6 (0.025 *N* with respect to potassium thiocyanate).

PROCESS. *Solution.* Ash 0.5 to 2 g. of the plant sample below 450°, take up in 1 to 2 ml. of *hydrochloric acid,* evaporate to dryness, take up in a few milliliters of *1.0 N hydrochloric acid,* and transfer with 20 to 30 ml. of water to a 50-ml. beaker. Adjust to a *p*H between 4 and 5 by means of *dilute ammonium hydroxide,* using the glass electrode, filter, and wash, thus removing practically all the iron and aluminum, then evaporate to dryness on the steam bath. Take up the residue in 25 ml. of *0.1 N ammonium acetate solution.*

Polarographic Determination. Remove oxygen from the solution by a stream of pure *nitrogen.* Use in all determinations the same dropping mercury electrode and adjust the dropping rate to 2.0 to 2.6 seconds per drop. Measure the anode potential (1.01 volts) against the saturated calomel half-cell so as to correct the half-wave potential for it, one measurement usually being sufficient. Since

the adjustment of the *p*H is between 4 and 5, ferric iron and aluminum are practically removed. No interference from anions or cations ordinarily present in plant ash need be feared, even if the amount is abnormally high.

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9. ALCOHOLS

a. Ethanol and b. Methanol

See Part II, F and J3.

c. Glycerol

Glycerol, free or combined, is not only present as a natural constituent in fats, wines, and many other products, but also as an added constituent designed, as in dried prunes, to improve the appearance, as in shredded cocoanut, to secure a desirable texture, as in extracts, to furnish "body," or as in various products for special reasons that may not be in the interests of the consumer.

Methods for special products are given under Wines, and in other sections. The following methods are for general use after removal of the interfering substances.

Cuthill and Atkins Ceric Sulfate Volumetric Method.[1] This and the following method (Bradford Technical College) are as commendably brief in operation as they are in description.

Process. Reflux 10 ml. of the sample, or a solution of the sample containing approximately 0.025 M of glycerol, with 50 ml. of *0.1 N ceric sulfate solution* and 50 ml. of *2 N sulfuric acid* for 1 hour. Cool, add 1 ml. of *standard 0.01% xylene-cyanol FF solution in water*, and titrate the excess of ceric sulfate with *standard 0.1 N ferrous ammonium sulfate solution*.

Calculation. One molecule of glycerol reacts with 4 atoms of oxygen, which is the amount required to form tartronic acid, although no attempt was made to establish the formation of that substance.

Cuthill and Atkins Hypobromite Volumetric Method.[1] Reagent. *Hypobromite Solution*, about 0.1 N. Dissolve about 50 g. of potassium bromide in a mixture of 600 ml. of water and 50 ml. of 4 N sodium hydroxide solution. Add 3.5 ml. of bromine and dilute to 1 liter with water. Standardize against standard thiosulfate solution after acidifying with 2 N sulfuric acid and addition of potassium iodide. The solution, although more stable than that usually employed, loses 0.2 and 1.2% in 6 and 35 days respectively.

Process. Boil 10 ml. of the sample, or a solution containing 2.582 g. per liter, for 90 minutes with an excess of the *hypobromite solution* but not exceeding 30 ml. Add acid and iodide and titrate the excess with the *thiosulfate solution* as in the standardization of the reagent.

Calculation. The reaction is

$$C_3H_5(OH)_3 + 7O \rightarrow 4H_2O + 3CO_2$$

Fleury and Fatôme Periodic Acid Volumetric Method.[2, 3] These authors oxidize glycerol with periodic acid to formaldehyde and formic acid and destroy the excess of periodic acid with arsenious acid as follows:

$$CH_2OH \cdot CHOH \cdot CH_2OH + 2HIO_4 \rightarrow$$
$$2HCHO + HCOOH + 2HIO_3 + H_2O$$
$$H_3AsO_3 + HIO_4 -$$

Reagents. *Periodic Acid Reagent*, 0.1 M. Place in a 100-ml. flask 2.94 g. of $Na_3H_2IO_6$ or 3.04 g. of $K_2H_3IO_6$, add respectively 30 ml. or 20 ml. of 1.0 N H_2SO_4, agitate until dissolved, and make up to 100 ml.

Arsenious Acid Solution, 0.1 N, containing sodium bicarbonate. Prepare according to the description of Treadwell-Hall[4] as follows: Sublime from a porcelain dish onto a watch glass commercial arsenic trioxide. If arsenic trisulfide is present, forming a yellow sub-

limate, dissolve a new portion in hot 4 *N* HCl, filter off the yellow sulfide, allow the arsenic trioxide to settle on cooling, decant the mother liquid, wash the crystals of arsenic trioxide (As_2O_3) with water, and dry for 12 hours over calcium chloride. Dissolve 4.946 g. of the crystals and 15 g. of Na_2CO_3 in 150 ml. of water, transfer to a 1-liter volumetric flask, add 25 ml. of 1.0 *N* sulfuric acid, dilute at 20° to the mark, and mix. Standardize by using the solution for titrating 25 ml. of 0.1 *N* iodine solution diluted to 200 ml., with the addition of 2 ml. of 6 *N* HCl and then 2 g. of Na_2CO_3.

PROCESS. *Defecation.* Place 1.25 to 1.30 g. of *pulverized barium hydroxide* in a 50-ml. volumetric flask, add a suitable volume of the alcoholic liquor or the alcoholic extract of a solid food such as prunes or shredded cocoanut, containing no more than 40 ml. of glycerol, 0.35 g. of sucrose, or 0.17 g. of dextrose or invert sugar, shake, and cool in a refrigerator for 2 hours. Make up to the mark with ice cold *ethanol* and return to the refrigerator when the precipitate begins to form. After 15 to 24 hours, centrifuge and pipet 25 ml. of the clear supernatant liquid into a beaker. Precipitate the excess of barium with *20% sulfuric acid* or by passing a stream of *carbon dioxide* into the liquid. (Repeat on a new portion the barium hydroxide precipitation if no precipitate forms.) Add 25 ml. of water and evaporate to 20 ml. to remove the ethanol. Nearly neutralize with *0.1 N sodium hydroxide solution.* Without removing the precipitate of barium sulfate, proceed with the titration of the unknown as directed below.

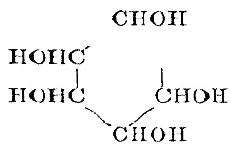
Blank Titration. Pipet into an Erlenmeyer flask 5 ml. of *periodic acid reagent,* add 5 to 10 ml. of *saturated sodium bicarbonate solution,* followed by exactly 15 ml. of *0.1 N arsenious acid solution* and 1 ml. of *0.1 N potassium iodide solution.* After allowing to stand 15 minutes, titrate the excess of arsenious acid with *standard 0.1 N iodine solution* delivered from a buret with fortieth-milliliter divisions. Designate the number of milliliters required as *C*.

Titration of Unknown. To the sample or a solution of the sample, add 5 ml. of *periodic acid reagent,* allow to stand at room temperature (16 to 22°), then proceed as in the blank titration. Designate the number of milliliters as C', which must be greater than C, since the amount of arsenious acid remaining after the reaction with the periodic acid is greater than after the blank titration.

CALCULATION. Expressed in milliliters of the 0.1 *M* solution, the periodic acid before the reaction with glycerol is $15 - C$, whereas after the reaction it is $15 - C'$; consequently the periodic acid consumed is $(15 - C) - (15 - C') = C' - C$. Furthermore, since 2 moles of periodic acid correspond to 1 mole of glycerol, or 1 ml. of a $M/10$ solution of periodic acid is equivalent to 1 ml. of $M/20$ solution of glycerol, and 4.6 g. per liter of glycerol are equivalent to 4.6 mg. per ml., it follows that the weight of glycerol (G) in the charge is $[(C' - C)/2] \times 4.6 = (C' - C) \times 2.3$ mg.

d. INOSITOL

Inositol, a sugar alcohol, although not a carbohydrate, has the same empirical formula ($C_6H_{12}O_6$) as dextrose; it is a hexahydroxyhexahydrobenzene:



The inactive form (meso-inositol) occurs in muscle, animal organs, and is widely distributed in the vegetable kingdom. It is a growth-promoting substance, which in yeast has sometimes been called biose I.

Fleury and Joly Sodium Periodate Volumetric Method.[5] The method was developed

at the Biological Laboratory of the School of Pharmacy, Paris. The reaction involved follows:

$$\underset{\text{Inositol}}{C_6H_6(OH)_6} + \underset{\text{Periodic acid}}{6HIO_4} = \underset{\text{Formic acid}}{6HCOOH} + \underset{\text{Iodic acid}}{6HIO_3}$$

REAGENTS. *Standard Arsenious Acid Solution*, 0.1 *N*. See Fleury and Fatôme Method under Glycerol above.

Standard Iodine Solution, 0.1 *N*, in 2 to 2.5% KI.

PROCESS. A. DEXTROSE ABSENT. *Treatment with Periodate.* To a known volume of the aqueous solution, containing 0.5 to 6 mg. of inositol, add 5 ml. of *0.1 M sodium periodate solution*, 2 ml. of *20% sulfuric acid*, and water to 50 ml. Allow to stand 24 hours at room temperature, then add a slight excess of *sodium bicarbonate*, 20 ml. of *0.1 N standard arsenious acid solution*, and 1 ml. of *20% potassium iodide solution*.

Titration. After 10 minutes at room temperature, titrate the excess of arsenic trioxide with *standard 0.1 N iodine solution* to a persistent yellow color.

CALCULATION. Subtract from the volume of 0.1 *N* iodine solution used that of a blank made on water. The difference (V) is the number of milliliters of 0.1 *N* periodic acid consumed and $V/2$ is the equivalent number of milliliters of 0.1 *M* periodic acid. Each molecule of inositol (mol. wt. 180) consumes 6 molecules of periodic acid, hence the weight of inositol in the solution analyzed is $(18 \times V)/(2 \times 6 \times 1000)$ g. $= 0.0015 \times V$ g.

B. DEXTROSE PRESENT. Two procedures are available: (1) direct determination of inositol by the periodic acid method, after destruction of the dextrose by heating with magnesium oxide, and (2) indirect determination by subtraction of the dextrose separately determined by the Bertrand copper reduction method from the sum of the inositol and dextrose obtained by the periodic acid method.

(*1*) *Direct Procedure.* Dissolve **1** g. of the sample in 30 ml. of water and destroy the dextrose by heating with 6 g. of *freshly calcined magnesia* on a boiling water bath for 30 minutes. Cool, add to the yellow-brown residue 20 ml. of water, shake, allow to stand 24 hours, filter on a Büchner filter, and wash thoroughly with 150 to 200 ml. of water. Concentrate the filtrate and washings by distillation under reduced pressure. Dry the residue over sulfuric acid, dissolve the inositol in boiling *80% ethanol*, and allow to crystallize in a refrigerator. Determine the inositol by the Periodic Acid Method as described above.

(*2*) *Indirect Procedure.* Determine the dextrose plus inositol by the Periodic Acid method, following the directions given above. Supplementing their somewhat vague directions, Fleury and Joly give the illuminating number of milliliters of 0.1 *M* periodic acid consumed for 1 ml. each of three solutions of dextrose and inositol in 30 ml. of solution respectively as follows: 2.05 ml. for 8 and 24 mg.; 1.96 ml. for 16 and 16 mg.; and 1.875 ml. for 24 and 8 mg. These figures might well be checked by the analyst and supplemented by results on solutions of 32 mg. each of dextrose and inositol separately treated.

Determine the dextrose by the Bertrand Copper Reduction Method as described under Dextrose, Part I, C6*a*. If the results fall outside of Bertrand's table, Fleury and Joly use Guillaumin's method.[6] Subtract the amount of dextrose found from the sum of the amounts of inositol and dextrose.

Smirnov Lead Method.[7] No details available.

Woolley Yeast Growth Turbidity Method.[8] Woolley (Rockefeller Institute) makes use of the indispensability of inositol for yeast growth. A series of cultures supplied with all the other essentials are treated with different amounts of yeast and the turbidity produced is compared with that of the unknown.

Apparatus. *Evelyn Photoelectric Colorimeter.*

Reagents. *Basal Medium.* Hydrolyze vitamin-free casein by autoclaving for 16 hours at 15 pounds pressure, with 7 *N* H_2SO_4, and remove the acid of the hydrolyzate with $Ba(OH)_2$. Adjust the filtrate to *p*H 6 with NaOH and mix an aliquot (= 2.5 g. of casein) with 100 g. of *dextrose,* 8.3 g. of NH_4NO_3, 4.2 g. of KH_2PO_4, 2.1 g. of $MgSO_4 \cdot 7H_2O$, 0.7 g. of $CaCl_2 \cdot 6H_2O$, and about 300 ml. of water, then autoclave at 15 pounds pressure for 15 minutes. Remove the precipitate that forms and to the filtrate add the non-dialyzable portion of 10 g. of *rice bran extract* (Vitab), made by dissolving rice bran extract in water and dialyzing against running water for 48 hours. Add the mixture of growth factors given below, followed by the concentrate of *Lucas' bios II* [9] equivalent to 10 g. of malt sprouts. Adjust the volume to 500 ml. and preserve with *toluene.* Prepare the growth factor by mixing *thiamin* 0.5 mg., *riboflavin* 0.5 mg., *vitamin* B_6 0.5 mg., *nicotinic acid* 1 mg., *choline chloride* 2.5 mg., *pimelic acid* 1 mg., *asparagine* 5 mg., *biotin* 0.05 mg., *sodium pantothenate* 0.5 mg., *uracil* 2.5 mg., and *adenine* 2.5 mg.

Organism. Maintain a Hansen No. 1 strain of Toronto yeast (*Saccharomyces cereviseae*) on slants of the *basal medium plus 10% aqueous extract of malt sprouts per 100 ml. of medium.* Transfer the organisms to a *liquid medium* of the same composition and allow to grow 24 hours at 30°. Collect the cells by centrifuging, wash three times with sterile *phosphate buffer,* and suspend in a volume of buffer 20 times that of the original culture. Inoculate each flask with 1 drop (0.05 ml.) of this suspension.

Process. *Solution.* Reflux a quantity of the sample expected to contain 20 γ of inositol or more in *18% hydrochloric acid* for 6 hours. For moist samples, use proportionately stronger acid. Evaporate to dryness, take up in water, neutralize with *sodium hydroxide solution,* filter, and adjust to a volume containing about 2 γ/ml. of inositol.

Inoculation and Incubation. Place 5-ml. portions of *basal medium* in a series of 50-ml. Erlenmeyer flasks and add the solution of the unknown covering 1 to 0.1 γ/ml. of inositol. Also prepare a series containing 10 to 0.05 γ/ml. of pure inositol. Add water to all the flasks to a final volume of 11 ml., autoclave the flasks at 15 pounds pressure for 15 minutes, inoculate each, and incubate in a water bath at 30° for 16 hours.

Turbidity Reading. Examine the contents of each flask for turbidity in an Evelyn photoelectric colorimeter.

Calculation. Compare the readings of the dilution of the unknown with a curve prepared from data obtained with the knowns, plotting colorimeter readings against readings on solutions of pure inositol containing from 0 to 5.0 γ/ml.

Examples. Corn (maize) 0.5, oats 1.0, alfalfa leaf meal 2.1, beef liver 3.4, beef heart 16.0, brewer's yeast 5.0, and whole milk 0.5γ/mg.

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[9] *J. Phys. Chem.* 1924, **28**, 1180.

10. VITAMINS

The methods are arranged in the usual order of capital letters and the results are expressed in terms of gammas per gram (γ/g.) so far as possible.

The occurrence and nutritional properties of the vitamins are treated in recent editions of works on biological chemistry and nutrition. The Weston-Levine Vitamin Chart by Roe E. Remington[1] (Medical College of South Carolina) in its frequent revisions gives excellent summaries. The chemical constitution of different members of the group together with historical notes appears in the introduction to each volume of the *Structure and Composition of Foods*, so far as the phenomenal progress in vitamin science between the publication of the several volumes permitted. It remains in this volume to give the most recent formulas at the head of the sections describing the methods for their determination.

Vitamins A (including carotene), D, and E (catch-word ADE), and also K are soluble in fat and fat solvents, those classed under B, also vitamin C, and doubtless others are soluble in water and slightly soluble in ethanol, but not readily, if at all, in the solvents characterizing the fat-soluble group.

CAROTENOIDS

See also Part II, A1 and B2.

Separation and Determination of Carotenoids. **Kuhn and Brockmann Methanol-Naphtha Colorimetric Method.**[2] The carotenoids are first partitioned into hydrocarbons and oxygenated compounds by their solubilities respectively in naphtha and 90% methanol, following the general plan of Willstätter and associates, then the members of the two groups are fractioned by adsorption in Tswett columns and elution, and their amounts determined by colorimetric analysis. Satisfactory recoveries were secured from solutions of the pure substances.

REAGENTS. *Naphtha.* Petroleum ether boiling between 70 and 80°.

Azobenzol Solution. Dissolve 14.5 mg. in 100 ml. of 96% ethanol (0.796 millimole per liter). This solution corresponds in color value to solutions of carotenoids in naphtha (b.p. 70 to 80°) as follows:

γ/ml.	Carotenoid	Formula	Mol. Wt.	Absorption Bands	
2.35	α-carotene	$C_{40}H_{56}$	536.5	478	447.5
2.35	β-carotene	$C_{40}H_{56}$	"	484	451
2.52	lutein	$C_{40}H_{56}O_2$	568.5	477.5	447.5
2.52	zeaxanthin	$C_{40}H_{56}O_2$	"	483.5	451
2.70	taraxanthin	$C_{40}H_{56}O_4$	600.5	472	443
2.70	violaxanthin	$C_{40}H_{56}O_4$	"	472	443
4.60	helenien	$C_{72}H_{118}O_4$	1047	477	447.5
4.60	physalien	$C_{72}H_{118}O_4$	"	483	451

Note that the color values are approximately proportional to the molecular weights.

Alcoholic Potassium Hydroxide Solution. Dissolve 5 g. of KOH in ethanol and make up to 100 ml. with the solvent.

PROCESS. *Extraction and First Separation.* Rub up 0.1 to 0.2 g. of the finely ground vegetable material, preferably dried over phosphoric pentoxide, in a porcelain dish with 40 to 50 ml. of *absolute methanol*, then with 40 to 50 ml. of *naphtha* added in small portions, centrifuging and filtering after each treatment. Grind animal tissues, such as liver, with sand previously to the addition of the methanol. Examine the residue with a lens and, if colored particles, sugges-

tive of carotenoids, are seen, repeat the process. Transfer the liquid to a cylindrical graduated separatory funnel of 120-ml. capacity, then add 4 to 5 ml. of water sufficient to reduce the strength of the methanol by about 10%. Shake vigorously and allow the liquids to separate. Draw off the lower layer into a 200-ml. graduated cylindrical separatory funnel and cautiously wash the naphtha solution 2 or 3 times with *90% methanol* until no further color is extracted. Shake the methanol solution with 1 or 2 portions of *naphtha* to insure complete removal of carotene, lycopene, or xanthophyl esters. Carefully collect the naphtha solutions in a small separatory funnel and wash 2 to 3 times with *90% methanol,* then add to the main naphtha extract. Add to the combined methanol solution the methanol used for washing.

A. Treatment of the Methanol Phase (Xanthophyls: Zeaxanthin, Zeaxanthin Ester, Lutein, Violaxanthin). If the methanol extract is colored green with chlorophyl, add 10 ml. of *2 N sodium hydroxide solution* and hold for 2 or 3 hours at room temperature. The presence of flavones is shown by a yellow to red-yellow color after the addition of the alkali. After the saponification of the chlorophyl is complete, add a little *naphtha,* dilute with water to double the volume, and shake violently. If an emulsion forms, it may be kept down by the addition of a little *2 N sodium hydroxide solution.*

In a few hours the naphtha layer separates as a clear, pure yellow solution. After repeated extraction with naphtha the diluted alcoholic layer contains all the xanthophyls. Wash the combined naphtha extract thoroughly (5 or 6 times) with water, otherwise a small amount of methanol will ruin the adsorption analysis. Transfer the washings to a volumetric flask of suitable volume for the colorimetric comparison and make up to the mark.

For the separation of *violaxanthin* from *lutein* and *zeaxanthin,* employ an adsorption tube 12 cm. long and 8 to 10 mm. wide, to the bottom of which is attached by a ground tapering joint a small bulb tube ending in a constricted opening. Prepare in the main tube a column of *strictly dry calcium carbonate* 7 cm. high by adding small quantities at a time and packing after each addition with a glass rod. Attach the adsorption tube thus prepared to a suction flask, turn on gentle suction, and pour an aliquot (or if necessary the whole) of the naphtha solution of the xanthophyls into the tube.

As soon as the solution has soaked the calcium carbonate, wash with pure *naphtha,* taking care that the calcium carbonate is always covered with the liquid. *Lutein* and *zeaxanthin* pass rather quickly through the column as golden yellow washed-out zones, whereas *violaxanthin* remains behind, forming a sharply defined yellow ring in the calcium carbonate. When the lutein and zeaxanthin have passed through, make the filtrate up to a definite volume in a volumetric flask or a graduated centrifuge tube with 0.1-ml. divisions, and determine the color values.

Elute *violaxanthin* with *naphtha* containing 1% of *ethanol.* In order to prevent too great a dilution of the dye, place in the bulb tube *adsorption grade fuller's earth* which adsorbs with avidity lutein and zeaxanthin. After these two xanthophyls are absorbed in the fuller's earth, remove the main tube, then dissolve the *lutein* and *zeaxanthin* from the bulb tube, which serves as a funnel, in a few milliliters of *ethanolic naphtha.*

Measurements of the adsorption bands serve to characterize the pigments in the fractions. The *violaxanthin* fraction in addition gives the characteristic reaction with *25% hydrochloric acid.*

B. Treatment of the Naphtha Phase (Carotene, Lycopene). Add to the naphtha solution of the first separation, containing

xanthophyl esters in addition to carotene and lycopene, an equal volume of *alcoholic potassium hydroxide solution* and heat at 40° for 3 hours. If the alcoholic alkali does not mix completely with the naphtha solution, add a little *absolute ethanol*.

After saponification of the esters, add water sufficient to reduce the strength of the ethanol to 20%; this insures practical freedom of the aqueous phase from carotene and lycopene. Separate the naphtha layer and wash repeatedly with *90% methanol* until no more color is removed, thus eliminating the chlorophyl that contaminated the naphtha phase after the first separation. Dilute the methanol solution of chlorophyl with water and shake out with *naphtha* in the same manner as directed for the first separation of xanthophyls and subject to the adsorption procedure.

Make an adsorption analysis of the naphtha layer of the second separation, containing *carotene* and *lycopene*, after thorough washing, using as adsorption material a mixture of 10 parts of *fuller's earth* (Merck) and 40 parts of *c.p. anhydrous aluminum oxide* (Merck). Follow in essential details the directions given above for xanthophyls. The carotene on subsequent washing runs markedly faster through the adsorption material than the *lycopene* which proceeds slowly as a light red ring. Remove the lycopene by eluting with *naphtha* containing 1% of *ethanol* and subject to colorimetric analysis.

Colorimetric Analysis. Make the color comparison in a micro colorimeter tube of 1-ml. capacity provided with a tight perforated stopper through which the tube of the adsorption cylinder passes, thus avoiding evaporation. Employ as the color standard *azobenzene solution* which is stated to be more satisfactory than a solution of potassium bichromate. Make the reading with daylight or a daylight lamp, using for greater accuracy a blue (cobalt) filter. Set the tube containing the standard at 10 mm. and adjust the tube containing the unknown diluted so that the depth of the liquid when matched will be between 4 and 20 mm. Within these limits the values of the solution of carotenoids may be regarded as conforming to Beer's law.

Decomposition Products of Carotenoids. Decomposition products due to oxidation or the action of acids if colored cause a considerable error. Those formed from the hydrocarbons are least troublesome. They are partly soluble in 90% methanol and consequently accompany the *xanthophyls* in the first separation. Another part present in the unsaponifiable matter of the naphtha phase is more easily adsorbed by the fuller's earth than lycopene and carotene and may be separated as the top layer of the chromatogram before eluting the *lycopene*. This phenomenon furnishes a useful test for oxidation products since they do not penetrate far into the column.

Carotene is much more resistant to acids than lutein. On treatment of carotene with *oxalic acid* under conditions that cause a change in color or intensity of the adsorption band of *lutein*, no change is evident.

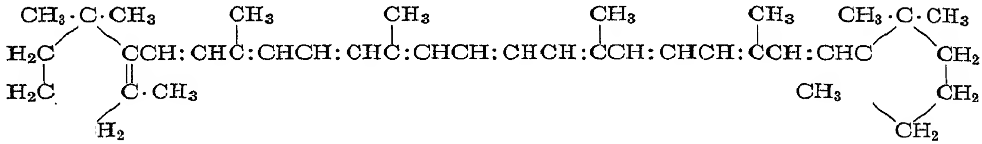
The decomposition of *xanthophyls* presents, as noted by Kuhn, Winterstein, and Lederer,[3] greater difficulties. A very small amount of *organic acids* causes marked changes in the deportment of *lutein*. The color of the solution is not so pronounced as that of *carotene* in naphtha solution or after saponification. There is no material change in the adsorption bands or adsorption deportment. *Lutein*, which is changed by oxidation in the air or by the gentle action of *chromic acid*, remains in the chromatogram on top of the calcium carbonate where it may be removed. *Pure lutein* is so tightly held by fuller's earth that removal is practically impossible. *Oxidized lutein* deports itself in the fractioning like the unchanged pigment and exhibits faint scarcely recognizable adsorption bands.

Carotene

Four members of the carotenoid group (α-, β-, and γ-carotene, also cryptoxanthin) form in the animal digestive system vitamin A and are known collectively as provitamin A. They are orange or yellow in color and may be grouped also with other carotenoids as natural vegetable colors. In combination with chlorophyl, which is blue-green, they produce a yellow-green composite color.

The carotene content of vegetables, fruits, cereals, and other foods varies greatly, carrots and garden peppers being particularly rich in potent forms of the provitamin. β-Carotene is by far the most common of the group and the total carotene content in many foods differs little from the β-carotene content.

The formula for β-carotene is characterized by its symmetry:



β-Carotene (Karrer *et al.*)

α-Carotene differs from the β-form in that there is an extra H in one of the terminal rings at the juncture with the chain and CH_3 instead of H_2 occurs at the adjoining angle, thus removing the double bond to a new position.

Carotene, as seen under the microscope in carrots and paprika, is in the form of yellow needles, prisms, and spindles. The commercial product is dark red and crystalline, insoluble in water, sparingly soluble in ethanol and naphtha, but readily soluble in chloroform, carbon bisulfide, and benzene.

Analytical Methods. See also Part II, A1 and 2, C1, and G7.

Deleano and Dick Dichromate–Iodide Volumetric Method.[4] It is claimed by the originators (Bucharest) that this little-known method is six to seven times more accurate than the colorimetric methods for the determination of carotene. The steps are simple and call for no unusual chemicals. The method depends on the partial oxidation of carotene by potassium dichromate and the iodometric determination of the oxygen consumed.

Process. *Extraction.* Reduce the sample by grinding or chopping to a fine pulp. Dehydrate a weighed portion by drying at a gentle heat or preferably by allowing to stand over sulfuric acid. Mix with *carbon bisulfide* or *naphtha,* allow to stand overnight, and decant the liquid onto a filter, pressing the residue. Repeat the treatment to insure reasonably complete extraction. Distil off the solvent on a steam bath.

Oxidation. To the extract in a flask, add 5 to 10 ml. of *glacial acetic acid* in small portions and dilute with 150 ml. of water. Add 10 to 20 ml. of *0.1 N potassium dichromate solution,* then 10 ml. of *hydrochloric acid,* reflux for 1 hour, and cool.

Titration. Add 1 to 2 g. of *potassium iodide* in 5 ml. of water, allow to stand 3 to 6 minutes, and titrate with *0.1 N sodium thiosulfate solution,* first to a light yellow color, then, after adding 5 ml. of *starch solution,* to the disappearance of the blue color, shaking vigorously. Carry along with the actual analysis two or more blank determinations.

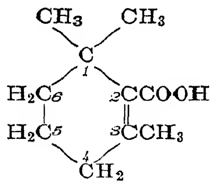
If less than 2.0 mg. of carotene is present in the solution, use half the quantities of the reagents given above and *0.01 N standard solutions.*

The distinctive reaction in the titration [5] follows:

$$K_2Cr_2O_7 + 6KI + 14HCl \rightarrow 8KCl + 2CrCl_3 + 7H_2O + 6I$$

CALCULATION. Subtract the milliliters of thiosulfate solution used for the titration in the actual analysis from that used in the titration of the blank and convert the difference into milligrams of oxygen by the factor 0.8 and the product into milligrams of carotene by the factor 1.0316. The combined factor is 0.825.

Complete oxidation of the side chain of carotene to carbon dioxide and water would require 40 atoms of oxygen, but the oxidation is incomplete and only 34 atoms of oxygen probably are consumed, the products being $4CH_3COOH$, $6CO_2$, $6CO$, and $2C_{10}H_{16}O_2$. The last-named substance is 1,1′, trimethyl-$\Delta^{2,3}$-cyclohexene-carboxylic acid with the following formula which differs from that of the terminal groups of the carotene formula in that a COOH group is attached at *2:*



In Deleano and Dick's experiments, only 32.5 atoms of oxygen were consumed and the factor 1.0316 was calculated on that basis by dividing 536.43 (mol. wt. of carotene) by 520 (mol. wt. of oxygen × 32.5).

Guilbert Sprague Dye Colorimetric Method.[6] For the determination of carotene in forage plants, Guilbert (University of California) omits the stages in the Schertz-Willstätter and Stoll procedure [7] for removing flavones which are later separated along with the chlorophyl by saponification.

APPARATUS. *Colorimeter or Nessler Tubes.*

REAGENT. *Sprague Standard Dye Solution.*[8] Dissolve in water 3.06 g. of Naphthol Yellow, after grinding to a paste with a little water, and 0.45 g. of Orange G crystals and make up to 1 liter. Dilute 5 ml. of this stock solution to 1 liter. Standardize against International Standard carotene (1 liter = 2.7 mg.) or against S.M.A. carotene, m.p. 166 to 168° (1 liter = 2.63 mg. of carotene).

PROCESS. *Solution and Saponification.* Mix 1 to 5 g. of the finely ground sample with 20 to 100 ml. of freshly prepared *saturated ethanolic potassium hydroxide solution,* and reflux for 30 minutes, washing down with *ethanol* any material adhering to the sides.

Ether Extraction. Cool, add 50 to 100 ml. of *ether,* shake for a minute or so, and allow to settle. Decant the ethanol-ether solution into a separatory funnel. Repeat the ether treatment twice with smaller quantities of the solvent, then add a small quantity of *ethanol.* Shake until the mass is broken up and extract with two or three portions of *ether,* which removes practically all the color. Discard the residue.

Through the combined ethanol-ether extracts in the separatory funnel, pour gently 100 ml. of water and allow to settle. Draw off the dilute ethanolic solution, which contains most of the chlorophylins and flavones, and reextract in another separatory funnel by gently shaking with *ether*, adding a little *ethanol* to remove any emulsion. Wash the combined ether extracts with water until chlorophylins and alkali are removed, pouring the first portions of water through the solution and down the sides, then shaking gently with the later portions until no color is given with *phenolphthalein*. Distil off the ether from the washed extract on a steam bath, removing the last few milliliters in a vacuum desiccator under diminished pressure.

Removal of Xanthophyls with Methanol. Dissolve the residue in 30 to 40 ml. of *naphtha,* transfer to a small separatory funnel and

extract the xanthophyls first with 5 or 6 equal volumes of *85% methanol*, then with *90% methanol* until no more color is removed. Wash the naphtha solution twice with water to remove the methanol, then filter into a 50- or 100-ml. volumetric flask through a small amount of *anhydrous sodium sulfate*, and make up to volume.

Color Comparison. Compare the color of the naphtha extract with the *Sprague standard dye solution* in a colorimeter or Nessler tubes.

Peterson, Hughes, and Freeman Spectrophotometric Method.[9] These workers have simplified the Guilbert procedure by dispensing with the ether extraction and all solvent evaporations. They extract the saponified mass directly with *naphtha* (Skelly solve B, b.p. 60 to 70°), following the details laid down by Guilbert for the ether extraction and washing with water. Chlorophylins, flavones, alkali, and xanthophyl are removed from the naphtha, following exactly the instructions given by Guilbert for the treatment of the ether and naphtha extracts.

Instead of determining the concentration by comparison with the Sprague dye standard, optical density measurements are made at wave lengths 4500, 4550, 4700, and 4800 Å. Absorption coefficients calculated for β-carotene in naphtha at these wave lengths are used in the following formula:

$$c = \frac{D}{kb}$$

in which c is the milligrams per gram (or grams per liter) of carotene, D is the reading of the spectrophotometer optical density, k is the extinction coefficient (specific transmissive index, or absorption index), and b is the thickness of the layer of solution in centimeters.

The extinction coefficients, as determined by Peterson *et al.* in Skelly Solve B are 4500 Å, 238; 4550 Å, 227; 4700 Å, 200; and 4800 Å, 212.

I. Brooke, Tyler, and Baker Modification.[10] In determining β-carotene at the Wirthmore Research Laboratory, Malden, Mass., multiple apparatus is used and as many as ten to twelve washings are eliminated.

Apparatus. *Mechanical Shaking Apparatus.*

Photelometer.

Process. *Hydrolysis.* Reflux for 30 minutes 2 g. of the finely ground alfalfa meal in a 100-ml. fat extraction flask with 15 ml. of *10% ethanolic potassium hydroxide solution* and 5 ml. of *ethanol* used to wash down the sides.

Naphtha Extraction. Cool quickly to room temperature, add 15 ml. of *naphtha*, cork, shake 2 minutes in the mechanical shaker, and filter through non-absorbent cotton into a separatory funnel. Repeat the shaking and filtering twice, add 10 ml. of *ethanol* to break up the residue, then extract with 15-ml. portions of *naphtha* as above, the total of six extractions usually being sufficient.

Removal of Alkali. Fill the separatory funnel with water and after allowing to stand for 15 minutes slowly drain off all the water layer, except about 1 ml. as a seal. Add 20 ml. of water, shake 2 minutes, draw off the water layer, then wash with 20-ml. portions of *89% methanol* until the methanol is water-white and free from alkali.

Desiccation. Finally filter through 1 g. of *anhydrous sodium sulfate*, dilute to 100 ml. with *naphtha*, and determine the concentration of β-carotene in the photelometer.[11]

II. Haagen-Smit, Jeffreys, and Kirchner Phosphoric Acid Spectrophotometric Modification.[12] This modification was developed at the California Institute of Technology, Pasadena, primarily for the separation of carotenes in canned pineapple. The phosphoric acid completely removes xanthophyls from the 85 to 90% methanol extract, but not lycopene; it does not separate α- from β-carotene.

PROCESS. *Saponification and Naphtha Extraction.* Treat the material for 12 hours at 3° with *30% potassium hydroxide solution in methanol* and extract with *naphtha* (60 to 70°).

Phosphoric Acid Treatment. Shake a 25-ml. aliquot of the naphtha extract with 4 to 5 ml. of *85% phosphoric acid* in a 25-ml. glass-stoppered graduate, thus removing the xanthophyl to the acid layer, to which it imparts a blue-green color.

Absorption. If the spectrophotometer is used, make the measurement of the absorption of the total carotenes in the xanthophyl-free naphtha extract at 440 to 450 mμ.

Munsey Dichromate Colorimetric Method.[13]

REAGENT. *Potassium Dichromate Solution*, 0.1%. The solution is used as a standard and a table is given showing the equivalent gammas per gram for colorimetric readings from 1.0 to 12.0 mm. of the standard.

Standardization. Dissolve 1 tube (0.1 g.) of S.M.A. carotene in about 2 ml. of $CHCl_3$ and precipitate with 20 ml. of methanol. Filter, wash with a few drops of methanol, and dry in a desiccator with diminished pressure for about 1 hour. Weigh very carefully 10 mg. of this purified carotene and dissolve in the smallest possible amount of chloroform, then dilute to 100 ml. with naphtha and make up 10 ml. of the diluted solution to 100 ml. with the solvent, thus obtaining a 0.001% carotene concentration. Pour the solution into the left-hand cup of the colorimeter and set the depth at 5 mm., then vary the depth of the right-hand cup, into which has been put the 0.1% potassium dichromate solution to be standardized, until the color matches. Adjust the right-hand cup to read 8.3 mm. by adding more dichromate or water. If the carotene solution is set at 4 mm., the dichromate solution reading should be 6.5 mm.

Other reagents as in the original method and the Peterson, Hughes, and Freeman modification, except that the *standard dichromate solution* is substituted for the standard dye solution.

PROCESS. The following details, in substance as given in the 1940 revision of the *Methods of the A.O.A.C.*, combine the features of the original method and the Peterson, Hughes, and Freeman modification with certain innovations, including a calculation table, supplied by Munsey and collaborators.

Saponification. Weigh 1 to 5 g. of the sample into a 200-ml. Erlenmeyer flask. For each gram add 20 ml. of freshly prepared *saturated ethanolic potassium hydroxide solution* and reflux for 30 minutes, washing down with *ethanol* from a wash bottle any of the material that collects on the sides of the flask.

Naphtha Extraction. Add 100 ml. of *naphtha*, shake for at least 1 minute, allow to settle, and decant the naphtha-ethanol mixture into a 500-ml. separatory funnel. Repeat the extraction twice with 25 ml. portions of *naphtha*, breaking up any masses by shaking with 10 to 15 ml. of *ethanol*, followed by 2 or 3 additional extractions with 20-ml. portions of the *naphtha*.

(An alternate procedure, economical of solvent, is to filter the saponified mixture directly through a sintered glass funnel, No. 3 porosity, and extract with small portions of the solvent until no more color is removed.)

Pour gently into the naphtha solution contained in the separatory funnel 100 ml. of water, draw off the alkaline ethanolic solution, and reextract three times by shaking with 30-ml. portions of *naphtha*, using two other separatory funnels. Combine the naphtha extracts and wash practically free from alkali with 50- or 100-ml. portions (about 10 or 5) of water, testing with *phenolphthalein indicator*. A trace of alkali will be removed later by subsequent washings.

Removal of Xanthophyl. Shake the naphtha solution with 25-ml. portions of *90% methanol* for 2 minutes, draw off the methanol, and repeat the treatment (6 to 12 times)

until the washings are colorless. Wash the naphtha solution twice with 50-ml. portions of water to remove methanol and adjust the volume by dilution or evaporation under reduced pressure to a suitable concentration for carotene measurement. Filter through *anhydrous sodium sulfate* and make up to a definite volume.

Color Reading. If a spectrophotometer is used, make optical density measurements at wave lengths 4500, 4700, and 4800 Å and determine the carotene concentration for each, using the absorption coefficients calculated for β-carotene in 60 to 70° and 40 to 60° naphtha respectively as follows: 4500 Å, 238 and 243; 4700 Å, 200 and 207; 4800 Å, 212 and 212.

Carotene from Potassium Dichromate (Munsey)

$K_2Cr_2O_7$ 0.1%	Carotene	$K_2Cr_2O_7$ 0.1%	Carotene
mm.	γ/g.	mm.	γ/g.
1.0	0.5	6.6	4.1
1.2	0.7	6.8	4.2
1.4	0.8	7.0	4.3
1.6	0.9	7.2	4.5
1.8	1.0	7.4	4.6
2.0	1.2	7.6	4.7
2.2	1.4	7.8	4.8
2.4	1.5	8.0	4.9
2.6	1.6	8.2	5.0
2.8	1.7	8.4	5.2
3.0	1.8	8.6	5.3
3.2	2.0	8.8	5.4
3.4	2.1	9.0	5.6
3.6	2.2	9.2	5.8
3.8	2.3	9.4	5.9
4.0	2.5	9.6	6.0
4.2	2.6	9.8	6.1
4.4	2.7	10.0	6.3
4.6	2.8	10.2	6.5
4.8	2.9	10.4	6.7
5.0	3.1	10.6	6.8
5.2	3.2	10.8	6.9
5.4	3.4	11.0	7.1
5.6	3.5	11.2	7.3
5.8	3.6	11.4	7.4
6.0	3.8	11.6	7.5
6.2	3.9	11.8	7.6
6.4	4.0	12.0	7.8

If a visual colorimeter is used, put the solution of the unknown in the left-hand cup, set the scale at a suitable depth in centimeters and adjust the *dichromate solution* in the right-hand cup to correspond in tint.

Calculation. From the table obtain the gammas per gram of carotene and the following formula to obtain the gammas per gram (C) in the sample:

$$C = \frac{c \times m}{g \times d}$$

in which c is the gammas per gram of carotene from the table, m is the millimeters of solution, g is the grams of sample, and d is the depth of the sample in centimeters.

Peterson Modification.[14] As practiced at the Kansas Agricultural Experiment Station, *ethanolic potassium hydroxide* (10%) serves for the extraction, *naphtha* (Skelly solve B) for the displacement of the ethanolic potassium hydroxide, *90% methanol* for the removal of extraneous pigments (chlorophylins, flavones, and xanthophyls), and *dichromate* for the color comparison.

Apparatus. *Colorimeter.*

Process. *Extraction.* Weigh 1 to 5 g. of the dry plant material, or 4 to 10 g. of the fresh, into a 250-ml. Erlenmeyer flask, add 100 ml. of freshly prepared *10% ethanolic potassium hydroxide solution*, and reflux for 30 minutes, rinsing down the sides with *ethanol.* Cool, filter through a sintered glass filter funnel, applying suction only when most of the liquid has passed through. With fresh material, grind the residue with *sand* at this stage and digest further with the *ethanolic potassium hydroxide solution.*

Naphtha Treatment. Wash the residue alternately with 25-ml. portions of *naphtha* and

absolute ethanol until the filtrate comes through clear, using suction only when there is liquid over the sediment. In certain cases stir the material on the funnel plate to secure better contact. Transfer the filtrate to a 500-ml. separatory funnel, pour gently 100 ml. of water through the ethanol-naphtha solution, draw off the alkaline ethanol-water solution, and reextract three times with 30-ml. portions of *naphtha*, using two other separatory funnels.

Methanol Treatment. Wash the combined naphtha extracts by shaking with about 5 portions of 30 ml. each of *90% methanol* and reextract the first portion with 50 ml. of *naphtha*. Remove the methanol from the naphtha solution by washing once with 50 ml. of water and filter through paper containing a small amount of *anhydrous sodium sulfate*.

Color Reading. Make the filtrate up to a definite volume and determine the color value by comparison with *0.1 or 0.036% standard potassium dichromate solution* in a suitable colorimeter.

Bickoff and Williams Direct Aluminum Oxide Chromatographic-Colorimetric Method.[15] Studies carried out at the Western Regional Research Laboratory, Bureau of Agricultural Chemistry and Engineering, Albany, California, led to the development of a method by which carotene in vegetable oils is separated, without saponification, from other chromogens such as xanthophyl and storage oxidation products of carotene.

Apparatus. *Evelyn Photoelectric Colorimeter*, with 440-mμ filter.

Tswett Tubes, 11 mm. inside diameter. Pack with 12 g. of activated aluminum oxide to the height of about 10 cm. by gentle suction and tamping with a glass rod flattened at the end. Cover with about 1 g. of anhydrous sodium sulfate.

Reagents. *Activated Aluminum Oxide* (Aluminum Ore Company, East St. Louis, Ill.). Determine the volume of eluent required for each lot of aluminum oxide by running into the column a 10-ml. aliquot of a naphtha solution of crystalline carotene containing 0.1 g. of oil per milliliter of solution, followed by washing with various amounts of the eluent. For example, the first three lots obtained by Bickoff and Williams required 45 ml. and the fourth lot required 65 ml. of eluent. Store in a dry container.

Naphtha (b.p. 30 to 60°). Alone, used as a solvent; mixed with 2% of acetone, used as an eluent.

Process. *Color Formation.* Run onto the column a 10-ml. aliquot of a naphtha solution containing 0.1 g. of the oil per milliliter of solution. After all the solution has passed into the aluminum oxide, simultaneously develop the column and elute the carotene with the required amount of *naphtha containing 2% of acetone*, without the use of pressure or vacuum.

Color Measurement. Dilute the eluate to 100 ml. and determine the carotene colorimetrically in an Evelyn photoelectric colorimeter provided with a 440-mμ filter.

Of the pigments that are thus determined together, α- and neo-β-carotene are more readily eluted than β-carotene. Determine the individual components by the special methods described in this section.

α- and β-Carotene

Although for many purposes results on the total carotenes meet the requirements, especially since the α-form is commonly present in much the smaller amount, a reliable method of separate determination is a distinct addition. See Buxton Method for Yellow Corn, Part II, A1.

Shrewsbury, Kraybill, and Withrow Photoelectric Photometric Method.[16] The method and the instrument employed originated at Purdue University.

Apparatus. The *Photoelectric Photometer*, described by Withrow, Shrewsbury, and Kraybill.[17]

PROCESS. *Separation and Purification.* The technique was originally designed for the examination of the two carotenes as separated and purified by the method described by Miller [18] as follows.

Dissolve a preparation of mixed α- and β-carotenes (S.M.A. Corporation, Cleveland, Ohio) in *light naphtha* and chromatograph in a column of *calcium hydroxide* 10 cm. high and 7.5 cm. wide. Separate the layers of the α- and β-forms and elute with *2% methanol in naphtha.* Make a final separation by again passing the carotenes through *calcium hydroxide,* elution, concentration at low temperature *in vacuo,* crystallization, and drying in a desiccator. Store in evacuated ampules.

If the material is a fresh or dry natural vegetable product, extraction and purification of the carotenes is essential, the procedure being adapted to the nature and amount of extraneous substances.

Preparation of Solution. Weigh out 10 to 15 mg. of the carotene preparation on a micro balance and dissolve in a suitable vol-

VALUES OF α- AND β-CAROTENES AT VARIOUS CONCENTRATIONS (WITHROW, SHREWSBURY, AND KRAYBILL PHOTOELECTRIC PHOTOMETER)

Carotene	α-Carotene		β-Carotene		Carotene	α-Carotene		β-Carotene	
γ/ml.	− log T	α	− log T	α	γ/ml.	− log T	α	− log T	α
0.4	0.079	196.8	0.080	199.6	3.0	0.556	185.4	0.578	192.8
0.5	.098	196.4	.100	199.4	3.1	.573	184.8	.596	192.4
0.6	.118	196.1	.120	199.1	3.2	.590	184.3	.615	192.1
0.7	.137	195.6	.140	198.8	3.3	.606	183.7	.633	191.8
0.8	.156	195.3	.160	198.7	3.4	.623	183.2	.651	191.4
0.9	.175	194.9	.179	198.4	3.5	.639	182.6	.669	191.1
1.0	.194	194.5	.198	198.2	3.6	.656	182.1	.687	190.7
1.1	.214	194.1	.218	198.0	3.7	.672	181.6	.704	190.4
1.2	.232	193.7	.238	197.7	3.8	.688	181.1	.721	190.0
1.3	.251	193.3	.258	197.5	3.9	.704	180.5	.740	189.7
1.4	.270	192.7	.277	197.2	4.0	.720	180.0	.757	189.3
1.5	.289	192.4	.297	197.0	4.1	.736	179.5	.776	188.9
1.6	.307	192.0	.315	196.7	4.2	.751	178.8	.792	188.6
1.7	.326	191.5	.334	196.4	4.3	.767	178.3	.809	188.2
1.8	.344	191.2	.353	196.1	4.4	.782	177.7	.826	187.8
1.9	.362	190.7	.372	195.9	4.5	.797	177.2	.843	187.4
2.0	.381	190.3	.392	195.6	4.6	.813	176.7	.860	187.0
2.1	.399	189.8	.411	195.4	4.7	.827	176.0	.877	186.6
2.2	.416	189.3	.431	195.1	4.8	.842	175.5	.894	186.2
2.3	.434	188.9	.448	194.8	4.9	.857	174.9	.910	185.8
2.4	.452	188.4	.467	194.5	5.0	.872	174.3	.927	185.4
2.5	.470	188.0	.486	194.2	5.1	.886	173.7	.944	185.0
2.6	.488	187.5	.504	194.0	5.2	.900	173.1	.960	184.6
2.7	.505	187.0	.523	193.7	5.3	.914	172.4	.976	184.2
2.8	.522	186.5	.542	193.4	5.4	.928	171.8	.993	183.8
2.9	.539	185.9	.560	193.0	5.5	.942	171.3	1.009	183.5

ume of *heptane*. Dilute five to eight measured portions of the solution so as to obtain concentrations differing by 0.5 mg. beginning with 0.5 mg.

Reading. Immediately after preparing the solutions, take the readings in a 1-cm. (2-cm. if the color is faint) cell. If a visual instrument is used, 2- to 10-cm. tubes or cells may be required. Take the reading of α-carotene at 447.5 or/and 475 mμ and of β-carotene at 455 or/and 480 mμ, which are the wave lengths of maximum absorption.

Calculation. Find in the table opposite, by Withrow *et al.*, the concentration (gammas per milliliter) corresponding to $-\log T$ or calculate by the following formula:

$$1000t$$

in which c is the concentration of carotene (γ/ml.), t is the $-\log$ of transmittancy of T, α the specific absorption coefficient, and l is the depth (or width) of the tube or cell in centimeters. The table renders unnecessary plotting of curves with wave lengths in millimicrons as abscissas and transmittancies as ordinates, or with carotene concentration (γ/ml.) as abscissas and α as ordinates, as carried out by Shrewsbury *et al.*

If the $-\log T$ corresponds closely with the gammas per milliliter as given in the table for either α- or β-carotene, purity is established.

For concentrations between 0.5 and 2.5 γ/ml., the value 192.2 for α-carotene and 196.8 for β-carotene may be used with an error of only 3 and 2% respectively.

β-Carotene and Neo-β-Carotene

Beadle and Zscheile Chromatographic-Spectrophotometric Method.[19] Studies made at Purdue University by the above authors brought out that 79 to 90% of the carotene of the vegetables consists of the β-form. The calculation and terminology are those of Comar and Zscheile. Both papers should be consulted for full details.

Process. *Adsorption*. The two isomeric forms of β-carotene form a homogeneous zone on magnesia, but the neo-β-form is less strongly adsorbed than the β-form on alumina.

Spectroscopy. The specific adsorption coefficients in hexane solution employed as analytical constants for β- and neo-β-carotene respectively are 4850 Å, 200 and 81.3, 4780 Å, 228 and 132, and 4360 Å, 196 and 196 liters per g. cm.

If the two β-carotenes are the only pigments present, calculate the total pigment concentration (C) from the following equation:

$$\frac{\log_{10} I(4360)}{196}$$

If, however, it is desired to determine each pigment simultaneously, combine

$$\log \frac{I_0}{I} - \alpha_1 C$$

(obtained by the simultaneous solution of equations 2 and 6 of Comar and Zscheile) and

$$\frac{C_2}{C} \times 100 = \text{per cent of component 2}$$

which gives

$$\frac{\log \frac{I_0}{I}}{\alpha_2 - \alpha_1} \times 100 = \text{per cent of component 2}$$

At 4780 Å, the last becomes

$$\frac{\frac{I_0}{I(4780)} \Big/ C - 132}{96} \times 100 = \text{per cent of component 2}$$

In the foregoing equations, C and C_2 are total concentration and concentration of

ping the flask for several seconds at the end in a water bath at 70°. Discontinue the suction but continue running in the *nitrogen* until a slight pressure of gas is obtained. Dilute the residue with *chloroform* and make up to 25 ml. in a volumetric flask. Remove 11 aliquots of 0.15 to 1.0 ml. to absorption test tubes by a micro pipet, dilute each to 1 ml. with *chloroform*, and stopper.

Color Comparison. Adjust the light intensity of the colorimeter so as to read 100 when a blank containing 1 ml. of *chloroform* and 9 ml. of the *modified Carr and Price reagent* are placed in the apparatus. Place successively each tube in the apparatus and add 9 ml. of the *reagent* from an automatic pipet. Read the galvanometer at the point of temporary stability, using filter No. 620, correct the reading, and record the corrected values of $2 - \log G$.

B. β-Carotene. *Preparation of Standard.* Dilute 12 aliquots of 0.10 to 2.00 ml. of the standard solution of pure β-carotene in naphtha (b.p. 68 to 70°) in duplicate to 10 ml. in volumetric tubes with naphtha. Determine the exact concentration of these aliquots with a universal spectrophotometer, using the value 2290 for the extinction coefficient $E^{1\%}_{1\text{ cm.}}$ (475 mμ).[26] Read the same series of solutions on the scale of the photoelectric colorimeter against a blank of pure naphtha, using filter No. 440. Record the galvanometer readings and the value of $2 - \log G$ corresponding to the corrected galvonometer readings.

Solution of Sample. For the determination of β-carotene in the chloroform solution of the material, measure the same amounts as of the stock solution in naphtha, evaporate to dryness, dissolve the carotene in *chloroform*, and dilute to the same volumes as described above. Pipet in duplicate 12 aliquots of 0.10 to 2.00 ml. as before. Read the solutions against a blank of *pure chloroform* with filter No. 440 and determine the values of $2 - \log G$.

C. Correction for β-Carotene in Vitamin A. Measure with a micro pipet aliquots of 0.10 to 0.70 ml. of the stock solution of pure *β-carotene in chloroform* containing 14.1 γ/ml. Dilute each to 1 ml. in absorption test tubes. Place each tube successively in the colorimeter, add 9 ml. of the *modified Carr and Price reagent* from an automatic pipet, and determine the value $2 - \log G$, using filter No. 620. For the blank, add 1 ml. of *chloroform* to 9 ml. of the *reagent* in an absorption test tube.

Note. Tompkins and Bolomey[27] (Stanford University) have shown that, if *methylene chloride* is used as a solvent for the oil of soupfin shark liver, vitamin A may be determined directly in an aliquot, without saponification; also that oil and vitamin A may be determined simultaneously.

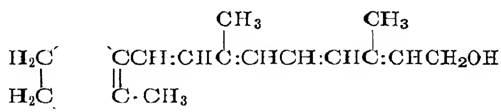
Vitamin A

See also β-Carotene and Vitamin A above.

No chemical name has been generally accepted for the anti-ophthalmia and anti-infection vitamin which now has only an alphabetical designation. A suitable name is not hard to find: *carene* would indicate relationship to carotene; *karrene* or *karrerene*, in addition, would suggest the name of Karrer.

Ready-formed vitamin A is especially abundant in fish liver oils from which concentrates are prepared. Other potent sources are mammalian liver, butter, cheese, and eggs.

During the formation of vitamin A from β-carotene by hydrolysis, the molecule is split in half and one molecule of water is added:



Vitamin A (Karrer, Morf, and Schöpp)[28]

This fat-soluble vitamin was isolated by Baxter and Robeson [29] as yellow prisms melting at 63 to 64°.

Chemical Methods. See also Part II, G1, and G7.

Lindholm,[30] in an extensive paper, compares results on vitamin A by biological, colorimetric, and spectrographic methods. He found that the acidity of the oil does not materially influence the results, as determined by the spectrograph, but that for very dark-colored oils the determination by both the colorimetric and spectrographic methods must be made on the unsaponifiable matter.

Holmes, Black, Eckler, Emmett, Heyl, Neilson, and Quinn [31] recommend that the E value of a reference oil, such as U.S.P. XI be determined spectrographically in each laboratory by the instrument there in use, and that the potency per gram of an oil in question be calculated by the following formula:

$$P = \frac{1.61 \times 1875 \times E'}{E}$$

in which P is the potency of the oil in terms of U.S.P. XI units, E' is the E value of the oil in question, and E is the E value of the U.S.P. XI oil, both as determined in the same laboratory using the Hilger vitameter.

Carr and Price Antimony Trichloride Colorimetric Method.[32] Although several marked color reactions characterize other vitamins, the blue color with antimony trichloride has had no persistent rival.

Apparatus. *Lovibond Tintometer* or *Hellige Comparator.*

Reagent. *Carr and Price Reagent.* Wash $SbCl_3$ with a little $CHCl_3$ (ordinary B.P. chloroform contains 2% of ethanol) and dry with the filter paper. Dissolve 30 g. of the purified salt in $CHCl_3$ and make up to 100 ml. with the same solvent. Allow to stand until clear, then decant.

Process. *Solution.* Dissolve in chloroform 20 g. of the oil, 20 g. of the ether extract from a known amount of animal or vegetable food, or the unsaponifiable matter from 20 g. of the oil or ether extract, and make up to 100 ml. with chloroform.

Color Comparison. Pipet 0.2 ml. of the chloroform solution of the unknown and 2 ml. of the *Carr and Price reagent* into a 1-cm. cell of the Lovibond tintometer and determine the color value by comparison with the colored slides which have been calibrated against a solution of the pure vitamin in like volume and treated in like manner as the unknown.

The Hellige comparator, employing colored disks, is also suitable, provided larger but proportional volumes are used. Direct comparison with the standard solution may be made in any ordinary colorimeter or Nessler tubes.

Calculation. To obtain the weight of vitamin in 1 g. of the sample, multiply the color value as determined by colored slides or disks by the factor representing the color unit. If the unknown was compared directly with the standard solution, divide the volume of that solution by the corresponding volume of the unknown and multiply the quotient by the weight of vitamin in the standard solution.

Notes. Coward, Dyer, Morton, and Gaddum [33] give directions practically identical with those of Carr and Price, but erroneously attribute the method to Rosenheim and Drummond.

In applying the test to butter, ice cream, edible fats and oils, and other food products, McCarley [34] warns that certain oil-soluble dyes give violet to carmine colors with the Carr and Price reagent.

Simmonnet, Busson, and Asselin [35] extract vitamin A and carotene from animal tissues as follows. Pulp 50 to 100 g. of the material, transfer at once to 5 volumes of *acetone*, store overnight in the refrigerator, filter rapidly through a Büchner funnel, and evaporate the filtrate. Treat the residue with an equal volume of *anhydrous ether* for 1 hour, sepa-

rate the extract, dry with *anhydrous sodium sulfate*, filter, and distil the ether. Take up the residue in a small amount of *anhydrous ether*, filter, evaporate, and weigh the dry extract.

Rosenthal and Erdélyi [36] sought to increase the permanence of the blue color by the addition of *pyrocatechol* to the reagent. Later Rosenthal, collaborating with Erdélyi, Weltner, and Szilárd,[37] substituted *guaiacol* for pyrocatechol; Willstaedt,[38] however, found that the reaction with this modification of the reagent was not specific.

French [39] found that phosphoric acid seemed to slow down the rate of fading of the blue color, but the effect was only apparent since the formation of a slight cloudiness tended to offset the fading. The end-point was determined by the Cenco photelometer used in conjunction with filter No. 4.

Evers [40] states that, although the results on the unsaponifiable matter of cod liver oil agree better with biological values than those on the oil, the latter are sufficiently accurate for commercial purposes.

Rygh [41] notes the presence in the unsaponifiable fraction of a substance, apparently a fatty acid, that decreases the blue color in the Carr and Price test.

Further studies on the Carr and Price reaction with the unsaponified oil, producing a purple-blue color, and with the saponified oil, producing a green-blue color, led Emmerie [42] to the conclusion that the cod liver oil acids, separated as a red-brown oil, caused the inhibition.

Zechmeister, Cholnoky, and Ujhelyi [43] dissolve the sample in *benzene* or *naphtha* and absorb vitamin A from the colorless solution on a *calcium hydroxide column*, then apply the antimony trichloride test directly to the column, after pushing it out of the cylinder, or after elution.

Meunier and Raoul [44] observed that the retardation of the Carr and Price reaction, in the presence of acetic anhydride, is more rapid for vitamin A_1 than A_2, in the absence of non-saturated fatty acids. They also found that the electric photometer readings, together with the inversion of the absorption values with the red and yellow filters, serves to distinguish the two forms easily.

I. Moore-Davies Colorimetric Modification.[45] The authors (Cambridge University) proceed with liver as follows.

Apparatus. *Lovibond Tintometer.*

Process. *Solution.* Mince with scissors 5 g. of the sample and treat in a 50-ml. beaker with 10 ml. of *5% aqueous potassium hydroxide solution*, as first adopted by Rosenheim and Webster.[46] Transfer to a 50-ml. Erlenmeyer flask and digest at once (or after keeping in cold storage, corked) in a steam oven until the solution is complete. Transfer to a 100-ml. separatory funnel, shake vigorously first with 5 ml. of *ethanol* for 10 seconds, then with the addition of 50 ml. of *ether* for 10 seconds. After discarding the aqueous layer, add 5 ml. of water, shake again vigorously, draw off the aqueous layer, wash the ether solution by gentle agitation with 50 ml. of water, and filter the ether fraction through a layer of *anhydrous sodium sulfate* in a sintered glass funnel into a wide-neck 100-ml. flask, washing the sulfate with a little *ether*. Evaporate the ether rapidly on a water bath, using suction.

Color Reaction. Dissolve the clean dry residue in 5 ml. of *chloroform* (more for high values). If moisture is present, add 1 to 2 drops of *acetic anhydride*. Pipet 0.02 to 0.5 ml. of the chloroform solution into a Lovibond cell or a test tube of 1-cm. bore checked against the cell. Make up to 0.5 ml. with *chloroform*, wash the pipet with *chloroform*, add 2 ml. of *Carr and Price reagent*, and determine the color value quickly in the Lovibond tintometer.

Calculation as Moore's Blue Units. In the formula

$$BU = \frac{b \times 2.5 \times 5}{5 \times c}$$

BU is Moore's blue units per gram of material, *b* is the blue reading (ignoring the yellow reading), *c* is the milliliters of chloroform solution, and 2.5 is the milliliters of the total reaction mixture. The other figures are of quantities as given above.

II. Notevarp and Weedon Spectrophotometric Modification.[47] The authors (Bergen, Norway) demonstrated that the blue solution obeys Beer's law, although the blue readings are not strictly proportional to the concentration, because of a physical phenomenon. Comparable results are obtained by adjusting the vitamin A concentration of the extract so as to secure a blue reading of about 6 which is accomplished by the use of the formula

$$c_6 = C_B \left(\frac{B}{6}\right)^{1/x}$$

in which c_6 is the concentration which will give a blue reading of 6, C is the concentration which has been found to give a blue value (B) of 6, using 0.2 ml. of extract and 2 ml. of the reagent, and x is the exponent 0.7 for oils or 0.8 for concentrates. The authors also established that $B = 5.5E^{0.7}$ for the maximum at 603 mμ (6030Å) wave length and $B = 8.5E^{0.8}$ for the maximum at 618 mμ (E being the absorption extinction), and that the blue value for the band at the longer wave length is 40 to 60% higher than for that at the shorter wave length.

The same authors found that by adding 0.1 g. of *bromine* to 1 liter of the *Carr and Price reagent* the inhibiting action on the 603-mμ band was almost completely removed and higher results, approaching those of the pure vitamin, were obtained. They express the relation of the biological value (*Bio*) to the extinction value at 603 mμ (E) by the following equation:

$$Bio = 6.5E^{0.75}$$

III. Dann and Evelyn Colorimetric Modification.[48] The notable features of this modification, developed at the medical departments of Duke University and McGill University, are the use of the Evelyn photoelectric colorimeter and the correction for carotene by special light filters and a special formula. The method combines the accuracy of a more expensive instrument and elimination of the human equation with convenience and rapidity. It is applicable to any fatty material containing vitamin A.

Apparatus. *Evelyn Photoelectric Colorimeter* (Fig. 12).[49] This is a single-photocell direct-reading photometer equipped with light filters. A beam of approximately monochromatic (filtered) light is directed upon a photoelectric cell connected to a galvanometer which records the intensity of the incident light directly on a scale of 100 divisions. An absorption cell interposed between the lamp and the photocell and the ratio of final and initial galvanometer readings measures the light transmission of the colored compound.

Reagent. *Modified Carr and Price Antimony Trichloride Reagent.* Dissolve 25 g. of $SbCl_3$ in $CHCl_3$ and make up to 100 ml. with the same solvent.

Process. *Saponification.* If the content of vitamin A is small (less than 10,000 I.U. per gram), saponify and use the unsaponifiable matter for the test, thus eliminating the interference of unsaturated fatty acid radicals with the Carr and Price reaction.

Boil a weighed amount of fat or oil (about 250 mg. for cod liver oil) in a small flask for 2 minutes with 2 ml. of *60% aqueous potassium hydroxide solution* and 10 ml. of *ethanol*. Pour the solution into 10 ml. of water contained in a separatory funnel, rinsing the flask with two 15-ml. portions of water and two 25-ml. portions of *ether*. Shake vigorously, draw off and discard the aqueous layer, then shake the ether solution vigorously with one 10-ml. portion of water and gently with two 25-ml. portions. Dry the ether solution by filtration through *anhydrous sodium sulfate* in a sintered glass diaphragm and wash the sulfate with 20 ml. of *ether*. Connect the flask with a

filter pump and evaporate the ether by immersion in warm water and the moisture by immersion for a moment in boiling water.

Solution. Dissolve the residue in 25 ml. of *chloroform*, pipet 0.25 ml. (for a trial) into a special photocolorimeter absorption test tube, and make up to 1 ml. with *chloroform*.

If the material is rich in the vitamin, weigh out a few milligrams without saponification, make up to a known volume with *chloroform*, and transfer an aliquot to the absorption test tube.

Color Comparison. Place filter 620 in the apparatus, turn the switch, adjust the light so that a galvanometer deflection of 100 divisions is obtained, using a blank tube containing 1 ml. of *chloroform* and 9 ml. of the *modified Carr and Price reagent* (or 10 ml. of the reagent if more convenient as it gives the same result). Replace the blank tube by the sample tube and run 9 ml. of the *reagent* rapidly down the side into it from an automatic pipet or buret. Read the position of temporary equilibrium (G_{620}) which the galvanometer spot reaches when the reagent and test solution become well mixed. If the reading is less than 20 or more than 70, set up another tube with less or more chloroform solution.

Calculation of L Value. In the absence of an appreciable amount of carotene, calculate as follows:

$$L \text{ value} \quad \frac{52.5}{M_{620}} \times \text{[illegible]}$$

in which $L_{620} = (2 - \log G_{620})$ and M_{620} is the weight in milligrams of the original material yielding the aliquot of unsaponifiable matter placed in the colorimeter tube.

Correction for Carotene. Carotene gives a color with antimony trichloride similar to that of vitamin A. When its presence is indicated by the yellow color of the chloroform solution, pipet 10 ml. of the solution into another colorimeter tube, replace filter 620 by 440, adjust the galvanometer so as to read 100 with a blank tube containing 10 ml. of chloroform only, then introduce the tube containing the solution of the material, and record the galvanometer reading (G_{440}). If the reading is less than 20, dilute the solution so as to get a reading between 20 and 70. The corrected value (L) is obtained from the following equation:

$$L = 52.5 \quad \text{[illegible]} \; M_{620} \quad \text{[illegible]} \; \frac{L_{440}}{M_{440}} \Big)$$

in which $L_{440} = (2 - \log G_{440})$ and M_{440} is the weight in milligrams of the original material yielding the aliquot of unsaponifiable residue corresponding to the reading G_{440}.

The following relations with other units are calculated from determinations made on 21 oils and 18 concentrates:

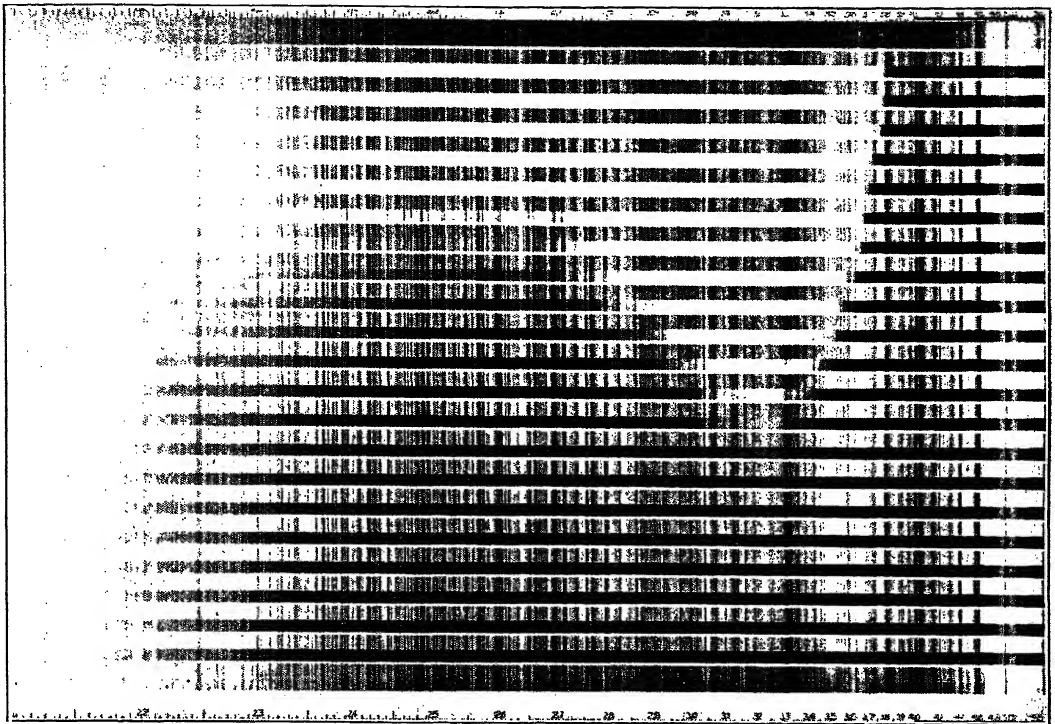
$$E(1\%, 1 \text{ cm.}, 328\ m\mu) = L \text{ value} \times (0.41 \pm 0.05)$$

$$\text{Blue value} = L \text{ value} \times (20 \pm 3.0)$$

Spectrographic Methods. See also Notevarp and Weedon Modification above.

Ultra-Violet Spectrographic Method. Absorption spectrograms in the visual range and less often in the ultra-violet region have long been of service in the study of the blood and other biological liquids. More recently ultra-violet absorption spectrograms have come to be regarded as the most exact basis for the quantitative determination of vitamins A and D. Methods for the determination of both vitamins, depending on visual absorption spectrograms of the colored liquid obtained with the Carr and Price reagent (antimony trichloride solution), are described under vitamin D, as are also the procedure employing the Carr and Price reagent, a color filter, and an ordinary colorimeter.

The cost of a quartz prism spectrograph, which may exceed that of all other optical equipment of a well-organized food laboratory, ordinarily limits the use of the ultra-violet spectrographic method to laboratories specializing in vitamin analysis.



Courtesy of Carl Zeiss, Inc., New York

FIG. 71. Absorption Spectra of Vitamin A Concentrate from Cod Liver Oil. 1 : 50,000 in Ethanol.

APPARATUS. *Quartz Prism Spectrograph.* See Introduction.

STANDARD CURVE. Prepare a solution in ethanol or ether of the pure vitamin or standard vitamin concentrate of suitable dilution and make a series of exposures (20 to 25), following the instructions accompanying the instrument or supplied by the manufacturer. Prepare a graph with wave lengths 2100 to 3800 Å as abscissas and extinction values (E) expressed as logarithms -1.0 to $+1.0$ as ordinates. Fig. 71 shows a series of spectra obtained with a standard vitamin A concentrate of 1 : 50,000 dilution in ethanol. The successive exposures represent fractions of the unknown solution, each accompanied by a standard spectrum, the same for each exposure, for comparison and location of the limits of the band. The fractionation is brought about by increasing (or diminishing) the light transmitted through the vitamin solution by successive change in the opening of a rotating sector, whereas the light yielding the standard spectrum remains constant because the opening is of the same size throughout.

The maximum absorption in the illustration lies at 3200 Å.

PROCESS. Dissolve a suitable amount of the oil or the ether extract in the *solvent* and make to the mark in a volumetric flask. Make a series of exposures, as above directed,

and compare with the standard curve. If the negatives show no curve repeat, using larger or smaller charge or dilution.

Calculation. Now that vitamins are firmly established on the chemical as well as the physiological basis, it is desirable so far as possible to express quantitative results in terms of gammas per gram rather than in units of potency, although the latter form still prevails in reports on fish liver oils and other pharmaceutical preparations rich in vitamins A and D. It is contended with reason that the unit system has the more practical value since both vitamins, or rather vitamin groups, owe their potency to more than one chemical substance of like or similar action and the unit represents the physiological resultant. A similar argument holds good, however, for the gammas per gram system, when the E value or other physical or chemical value is practically the same for the individuals of the vitamin group, as for example D_2 and D_3. The percentage system is universal for protein, fat, and carbohydrates, notwithstanding the composite nature of each, and our aim should be to bring vitamins into the same general system.

Note. The following equation, as given by Embree,[50] explains the significance of the symbols, often misunderstood:

$$E^{1\%}_{1\,\text{cm.}} = \frac{1}{c \times d} \times \log \frac{I_0}{I}$$

in which c is the concentration in grams per 100 ml., d is the depth of the optical cell in centimeters, I_0 is the intensity of light incident on the solution, and I is the intensity of the light transmitted by the solution.

I. Ewing, Vandenbelt, Emmett, and Bird Technique.[51] Ewing and Vandenbelt of the Michigan State College, collaborating with Emmett and Bird of Parke, Davis & Company, have made a critical study of the applicability of the spectrophotometric determination of vitamin A in fish liver oils. They ascertained the spread of the extinction coefficient $E^{1\%}_{1\,\text{cm.}}$ (1% solution in a cell 1 cm. thick) at 328 mμ wave length.

A. Spectrophotometric (Spectrographic) Procedure. Apparatus. *Spectrophotometric Set-Up*, consisting of (*a*) a *Bausch & Lomb sector photometer* with quartz optical system, (*b*) a *Bausch & Lomb medium quartz spectrograph*, (*c*) a *Bausch & Lomb 450 VA induction transformer* (ultra-violet radiation supplied by a condensed spark between tungsten steel electrodes), and (*d*) *Eastman No. 33 photographic plates* processed by No. D–1 developer at 18°.

Process. Dissolve a weighed portion in redistilled *isopropanol* (Eastman) and determine the spectrophotometric $E^{1\%}_{1\,\text{cm.}}$ value. Make the first photographic exposure 10 minutes after the preparation of the solution and other exposures immediately after. Process the plate under carefully controlled reproducible conditions and when dry mark the "isodensity" or "reversal" points on the glass side.

Calculation. Obtain the log (I_0/I) where I_0 is the incident light (100%) and I is the percentage of light transmitted at the wave length of the absorptive maximum. Calculate the extinction coefficient $E^{1\%}_{1\,\text{cm.}}$ by the formula

$$E^{1\%}_{1\,\text{cm.}} = \frac{1}{d \times c} \times \log \frac{I_0}{I}$$

in which d is the wave length of the light path through the solution in centimeters and c (percentage concentration) is 100 times the weight of the sample in grams divided by milliliters of solvent.

B. Vitameter Procedure. Apparatus. *Hilger Vitameter*. A ray of ultra-violet light from a copper arc passes through a filter transmitting principally copper wave lengths 3247.55 Å and 3273.97 Å, and then through a solution of fish liver oil in isopropanol. The ray impinges on a fluorescent screen as a line of light, approximately 1 cm. in length, which is compared visually with one from a

corresponding ray that does not pass through the solution. From the scale reading, calibrated as the log (I_0/I), calculate the $E^{1\%}_{1\,cm.}$ value.

Process. *Extraction.* Weigh 0.20 to 0.25 g. of a fish oil into a 50-ml. flask, add from a buret a volume in milliliters of *isopropanol* equal to 100 times the weight of the sample in grams, thus obtaining a 1% weight-volume concentration. Dilute further, using a calibrated pipet and small volumetric flask to give a vitameter scale extinction between 0.50 and 0.75.

Reading. Prepare 3 suitable dilutions and take 10 independent scale readings of extinction value for each.

Calculation. Divide the vitameter extinction values by the percentage concentrations of the corresponding solutions, and obtain the mean of all $E^{1\%}_{1\,cm.}$ values.

Examples. The above authors report spectrophotometric and vitametric values respectively on oils as follows: halibut 31.46, 32.20, halibut 50.12, 50.39, mixed 78.68, 79.82, mixed 128.6, 132.6, halibut 15.05, 15.12, mixed 39.85, 40.07, cod 1.589, 1.444, cod "nonsap." 1.346, 1.369 $E^{1\%}_{1\,cm.}$ mean values.

Conversion Factors. The mean factors for converting $E^{1\%}_{1\,cm.}$ to U.S.P. units per gram, as found by the two groups of workers, are for the spectrophotometer set-up 2152 and the vitameter 2122.

II. Coy, Sassaman, and Black Spectrophotometric Technique.[52] In the studies of cod liver oil in the vitamin laboratory of E. R. Squibb & Sons, New Brunswick, N. J., the following technique is employed.

Apparatus. *Spectrophotometer.* Judd Lewis Ultra-Violet Photometer, mounted in the frame of a 10 x 25 cm. Bausch & Lomb ultraviolet spectrograph. Calibrate the density scale of the lower sector over a range of 0.60 to 1.00 density values against three concentrations of potassium chromate solution.

Hilger Vitameter. For preliminary measurement.

Process. Weigh a suitable amount of the oil into a 100-ml. flask, dilute with *isopropanol*, take a preliminary vitamer reading, and adjust so as to give a match at a density reading of 0.50 to 0.95.

Place a similar concentration in one of the photometer cells with *isopropanol* in the compensating cell and expose a plate varying the aperture of the density scale from 0.15 to 0.95 by stages of 0.05. Read the matchpoint at 3280 Å to 0.05 density readings and also plot the absorption curve of the oil. Calculate the $E^{1\%}_{1\,cm.}$ value from Beer's law and expose a second plate, using two or three concentrations, each covering from six to nine exposures to ultra-violet light for not more than 1.5 minutes. The decrease in E value, due to irradiation, for exposures up to 3 minutes is well within the error of the instrument.

Expose a second weighing with two or three concentrations. If the extinction coefficients computed from the plates agree within 5% for the different weighings, no further data are necessary; if not, make a third weighing and exposure.

Examine the plates, when dry, on a viewing stand with a lens for density matchpoints at 3280 Å. If in doubt, project the plate on a screen with a Bausch & Lomb Balopticon projector and examine the projected image.

With low potency oils, carry out assays on both the oil and the unsaponifiable fraction.

Examples. Coy *et al.* in their first paper report $E^{1\%}_{1\,cm.}$ values and average conversion factors to U.S.P. XI biological units, based on biological value 3000 U.S.P. XI per gram respectively as follows: U.S.P. reference oil No. 1, whole 1.49 and 2010, unsaponifiable fraction 1.38 and 2180; cod liver oil, whole 0.58 to 2.75 and 2370, unsaponifiable fraction 0.55 to 2.33 and 2700; tuna liver oil 12 to 58 and 2180; halibut liver oil 18 to 93 and 2250.

In their later paper the average conversion factors, based on biological value 1700

U.S.P. XI per gram, are: U.S.P. reference cod liver oil No. 2, whole ($E^{1\%}_{1\,cm.}$ 0.85) 2000, unsaponifiable fraction ($E^{1\%}_{1\,cm.}$ 0.74) 2300; cod liver oil, whole 2070, unsaponifiable fraction 2170; tuna liver oil 1910, halibut liver oil 1960, and shark liver oil 1910.

Edisbury Spectrographic and Antimony Chloride Colorimetric Methods.[53] These methods are particularly adapted for concentrates, but may be used for fats and oils and other products after preliminary examination and preparation as indicated below.

APPARATUS. *Visual Spectrometer with Photometer*, for the color test.

Medium Quartz Spectrograph and Photometer (Spectrophotometer), taking 10 x 4 inch plates, for the ultra-violet examination.

Provided limitations inherent in the measurement instrument confined to 325 to 333 mμ are recognized and the instrument is adapted for photography and calibrated spectrometrically, the Hilger vitameter may be used as an inexpensive substitute for the spectrophotometer and is very nearly as accurate (about 3%).

REAGENTS. *Solvents.* Those usually used for ultra-violet work: *cyclohexane*, *ethanol*, *isopropanol*, etc.; avoid chloroform and ether. For measuring absorption at 620 mμ (characteristic of vitamin A when treated with $SbCl_3$ solution), it is essential to use cyclohexane or chloroform, alcohols being obviously excluded.

Modified Carr and Price Antimony Trichloride Reagent. Special care must be taken to remove $FeCl_3$. Purify a standard 20 to 25% solution of $SbCl_3$ in $CHCl_3$ by adding 0.1% of water and shaking vigorously. After allowing to stand overnight in the refrigerator, decant the clear reagent. Keep in colorless glass bottle (colored bottles yield iron) painted on the outside to exclude light.

PROCESS FOR CONCENTRATES (potency of 1000 to 200,000 I.U. or more per gram). *Solution.* Prepare a solution in pure *cyclohexane* and dilute as required. One per cent (*w/v*) is suitable for concentrates of 5000 I.U. per gram and concentrations in inverse proportions for materials of other potencies.

A. ULTRA-VIOLET ASSAY. Photograph the absorption spectra of duplicate portions in 2-stratum thicknesses that differ by about 10% (3 to 3.3 mm.). (Alternately, one portion can be diluted 10%, using an N.P.L. cylinder, and the same thickness employed again.) Determine the $E^{1\%}_{1\,cm.}$ 325 mμ value, which represents the total light absorption at 325 mμ due to the combined effects of vitamin A and the diluent oil, if any.

CALCULATION. The absorption of the refined and the deodorized oil often used as a diluent varies from sample to sample (range about 0.07 to 0.2 at this wave length) and must be deducted to obtain the true or net vitamin contribution. When available, a 1- to 1.5-mm. thickness of the diluent oil is convenient, the concentration being taken as 92% for purposes of calculation. If a sample of diluent oil is not available for the direct test, a standard deduction of 0.1 for the gross or total $E^{1\%}_{1\,cm.}$ 325 mμ value will thus bring the result to within 0.1 of the true figure. Such a correction is not required for values exceeding $E^{1\%}_{1\,cm.}$ 325 mμ = 10.

With further dilution the solution will be suitable for the vitameter reading.

In the usual routine, further examination is not required, but the color reaction forms a useful check, especially when the result is doubtful, and in unknowns provides a convenient approach from which appropriate conditions for ultra-violet assay can be calculated.

B. COLOR TESTS. *Color Comparison.* Use the same cyclohexane solution as for the ultra-violet assay. Estimate the Carr and Price blue value in the standard manner, but the solution must be diluted in an N.P.L. cylinder until, under accepted conditions of 0.2 ml. of solution and 2 ml. of reagent in a 1-cm. cell, the color matches 5 blue on the Lovibond scale plus the usual correction (e.g.,

$+1.5Y - 0.3N$). A low match gives high results and vice versa.

Spectrophotometer Color Test. Use the cyclohexane solution (1% for 5000 I.U. per gram) again, but otherwise follow the Morton technique [54] for essentials. Take intensity readings at absorption maximum 616 to 623 mμ and also at 580 mμ, where under normal circumstances the intensity is about one-half that at 620 mμ. Any marked deviation from a wave length of 620 mμ (e.g., 603 to 606 mμ, as seen in direct testing of cod liver oil and similar oils) or from a bright blue color, demands further investigation. A 2-cm. cell, matching at between 1.8 and 2.2 on the color intensity (log I_0/I) scale, as in the Carr and Price test, is essential for reliable results. Any cloudiness of the solution is removed by adding a drop of *acetic anhydride.*

C. Tests on Unsaponifiable Matter. Edisbury recommends the following procedure to unmask vitamin A in oleomargarine concentrates and fish oils of the cod-liver-oil type, although recognizing that unaccountable losses of 10 to 15% may occur.

Saponify the original mixture, dissolve 0.5 to 2.0 g. in 5 to 10 ml. of *cyclohexane,* and test as above, thus obtaining preliminary and confirmatory information. Boil duplicate 1-ml. portions with 0.2 to 0.4 ml. of *60% potassium hydroxide solution* and 5 ml. of *pure ethanol* for 5 to 10 minutes, dilute with 10 ml. of water, extract twice with 25 to 30 ml. of freshly distilled *ether*, wash the combined extracts with 10 ml. of water, 10 ml. of *dilute potassium hydroxide solution*, then three times each with 10 ml. of water, all at 30°. Remove the ether, add a few drops of *pure ethanol* to wet the residue, blow until dry two or three times with *nitrogen* or *carbon dioxide* at 100°.

Avoid strong sunlight and maintain anerobic conditions. There is no need for a reflux condenser, but the saponified mixture must not boil dry. Dissolve the unsaponifiable matter at once in 5 ml. of *cyclohexane* and proceed as before with the ultra-violet and color tests, and calculate as if the original oil were in solution.

Irradiation Methods. Although Chevallier and Chabre [55] believe that in general the vitamin A concentration of edible oils is proportional to the degree of absorption at 3280 Å, they observed that many oils contain pigments and free fatty acids with absorption bands respectively at 3300 and 3175 Å. They [56] employ a spectrophotometer in which advantage is taken of the constant ratio of (1) the continuous ultra-violet radiation emitted by a hydrogen tube and dispersed by a quartz monochromator, (2) the intensity of the fluorescent light of a potassium photoelectric cell covered with a fluorescent substance at the end of the monochromator, and (3) the photoelectric current generated by the fluorescent light. The same authors [57] note that carotenoids and more than 1% of free fatty acids introduce errors in the spectrophotometric determination of vitamin A; nevertheless a number of samples of cod liver oil were analyzed with results agreeing closely with those of the biological method.

Adam Hilger Ltd. in 1934 patented a process and special apparatus for comparing rays of about 3280 Å passed through the sample before and after estimating the vitamin content.

Chevallier [58] irradiates the alcoholic extract of the material by the rays from a hydrogen-vapor lamp and a Wood filter and plots the curve. Vitamin A is decomposed and its products at 3250 Å have an absorption value only two-thirds that of the original substance, whereas the values at 2900 and 3650 Å remain unchanged.

McFarlane and Sutherland [59] resort to intense ultra-violet irradiation whereby a 96% reduction of value E is effected. The values thus obtained are 20 to 30% lower than by absorption measurements on the unsaponifiable fraction.

Demarest,[60] in determining the content of

the vitamin, utilizes the destruction of vitamin A by a hydrogen arc light with the consequent 98% reduction in the extinction at 3280 Å. Long wave lengths in the ultraviolet region were more effective than short waves. Irradiation of a milk extract produced changes in the absorption spectrum radically different from those brought about by selective destruction of vitamin A.

Vitamins A_1 and A_2

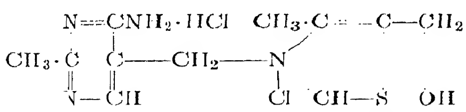
Huzita and Sakamoto [61] calculate the concentrations of vitamins A_1 and A_2 from the extinction coefficients (E) as obtained in the Pulfrich photometer, which are given for filters S 61 and S 66.6 as follows: A_1 1 : 0.258, A_2 1 : 0.508.

Thiamin

(Vitamin B or B_1)

Because of its action as a coenzyme in the oxidation of pyruvic acid from carbohydrates, thiamin is a preventive of certain nervous diseases such as beriberi and polyneuritis. It occurs in the largest amount in brewer's yeast, cereal embryos (germs), certain leguminous seeds, lean pork, and fish roe. It is present in good amount in various meats, eggs, milk, and leaf vegetables.

The molecule of thiamin hydrochloride (chloride) consists of a thiazole ring joined to a pyrimidine ring by a CH_2 group:



Thiamin Hydrochloride (R. R. Williams)

The vitamin is obtained as white crystalline powder, melting at about 245° with decomposition. It is soluble in water, slightly soluble in ethanol, but insoluble in fat solvents.

Analytical Methods. See also Part II, B1 and G1.

Kinnersley and Peters Diazotized Sulfanilic Acid Colorimetric Method.[62] See also Meunier and Blancpain Method below.

Apparatus. *Nessler Tubes* or *Colorimeter.*

Reagents. *Diazotized Sulfanilic Acid Solution* (Koessler and Hanke).[63] As needed prepare immediately before using as follows. Measure into a 50-ml. volumetric flask 1.5 ml. each of stock solutions of sulfanilic acid (4.5 g. of $C_6H_7O_3NS \cdot H_2O$ in 45 ml. of HCl and diluted to 500 ml.) and $NaNO_3$ (25 g. of 90% $NaNO_3$ dissolved in water and diluted to 500 ml.). Immerse in ice water for 5 minutes, then add 6 ml. more of the stock $NaNO_3$ solution, mix, cool again in ice water for 5 minutes, dilute to the mark, and return to the cooling bath. Do not use until cooled at least 15 minutes.

Alkaline Reagent. Dissolve 5.76 g. of $NaHCO_3$ in 100 ml. of 1 N NaOH and dilute with 100 ml. of water.

Taka-Diastase. Contains about 5% of phosphatase.

Process. In the original method, an aqueous solution of the vitamin was added directly to a mixture of the diazo and alkaline reagents and formaldehyde, but in the revised form as described below directions for preliminary removal of proteins from an acid ethanol solution, hydrolyzation of the phosphoric esters with phosphatase and extraction of the color with butanol are included.

Extraction. Weigh a suitable amount (e.g., 100 g. of oatmeal) of the finely ground product, add about 1000 ml. of boiling water, then 2 ml. of *hydrochloric acid* to bring the pH to 3.5, and heat for a few minutes to boiling. Remove to a boiling water bath and, after an hour or longer, add gradually *ethanol* sufficient to make 50% by volume. Cool overnight and filter on a Büchner funnel. Evaporate the filtrate to 150 ml. on the water bath, cool, and centrifuge.

Phosphotungstate Precipitation. To the

liquid, add *10% sodium phosphotungstate solution* at pH 6 in slight excess and acidify to pH 1.0 with *20% sulfuric acid.* Allow the phosphotungstate precipitate to settle 12 hours in a centrifuge tube, decant the liquid, then grind the precipitate with *barium hydroxide* and wash three times.[64] Precipitate the barium with *sulfuric acid*, filter, concentrate if necessary at pH 3, avoiding greater acidity during concentration, then remove any excess of sulfuric acid with *barium chloride solution*.

A. FREE THIAMIN. *Color Formation.* Place in a small test tube 0.1 to 0.3 ml. of a solution containing 10 to 20 γ of vitamin B_1 (acidity pH 6.0) and *30% ethanol*, after 1 minute add a mixture of 1.25 ml. of the *alkaline reagent* and 0.5 ml. of the *diazotized sulfanilic acid.* The ethanol is essential for the full development of the pink color.

Butanol and Acid Extraction. After allowing to stand for 2 hours or more, remove to a separatory funnel and extract the pink-colored solution with two successive portions of 2 ml. each of *butanol.* Extract the combined butanol solution once with 2 ml. and twice with 1 ml. of *0.005 N hydrochloric acid.* Bring to a pH of 6.5 by adding a small amount of *0.5% hydrochloric acid,* remove the colored acid phase, then add an equal volume of *ethanol.*

Color Reading. Compare the colored solution with a *standard solution* of the pure thiamin chloride treated in like manner, excluding strong light during the comparison.

NOTES. In the experience of the originators, reading in ordinary Nessler tubes is as accurate or even more accurate than in visual or photoelectric colorimeters. The results are accurate within ±5% for 20 γ of thiamin chloride. Reducing substances, such as cysteine or traces of sodium hydrosulfite, retard the color formation and diminish the color intensity. Most metals do not interfere, but exceptions are mercury (4 γ inhibits), silver (10 γ slightly detrimental), and copper (minute traces detrimental; maximum effect with an equivalent of 1 atomic weight per molecule) in solutions containing 10 γ of thiamin chloride. Panshina-Trufanova[65] states that the red color develops in 1 minute and persists at least 15 days. Diviatin[66] hastens the color formation by heating for 10 minutes in a water bath at 90 to 95°.

B. TOTAL THIAMIN. In the presence of phosphoric esters of thiamin (cocarboxylase, etc., as in yeast or yeast extracts), incubate at 38° for 1 hour 0.3 ml. of the solution with 50 ml. of *phosphate buffer* at pH 4.0 and 100 mg. of *taka-diastase* in 10 ml. of water, thus liberating the combined vitamin.

Proceed with the solution as directed under A above.

C. COMBINED THIAMIN. Obtain by difference.

Prebluda and McCollum Diazotized Amino-Acetophenone Colorimetric Method.[67] Both p-amino-acetanilid and methyl-p-amino-phenyl-ketone (p-amino-acetophenone) when diazotized with nitrous acid were found by Prebluda and McCollum (Johns Hopkins University) to yield with thiamin, under certain conditions, a characteristic purple-red compound which is stable and highly insoluble in water, but soluble in selective organic solvents. The method is based on the reaction with the second reagent.

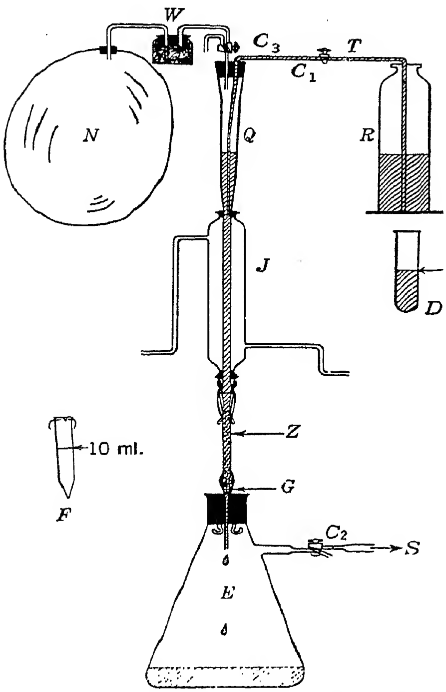
REAGENT. *Prebluda and McCollum Reagent.* (A) Dissolve 3.18 g. of p-amino-acetophenone (Eastman, No. 631) in 45 ml. of HCl (37%) and make up to 500 ml. in a glass-stoppered bottle. Keeps 6 months, away from strong light.

(B) Dissolve 22.5 g. of $NaNO_2$ in water and make up to 500 ml. in a glass-stoppered flask. Deteriorates in 1 month; store in refrigerator.

(C) Dissolve 20 g. of NaOH in 600 ml. of water, add 28.8 g. of $NaHCO_3$, and make up to 1 liter.

Mix daily 1 part by volume each of (A) and (B), allow to react 10 minutes in an ice

bath with agitation, then add 4 parts of (B) to the mixture, stir, and keep at 0 to 5° for at least 20 minutes. When diazotization is complete, store in a refrigerator. Place 20 ml. of the (A) and (B) mixture in a flask con-



Courtesy of the Authors and J. Biol. Chem. 1939, **127**, *523*

FIG. 72. Melnick and Field Thiamin Adsorption Assembly.

taining 275 ml. of (C) and stir 5 to 10 minutes, during which time the purple color that first forms disappears.

PROCESS. *Color Formation.* To 1 ml. of the solution of the sample adjusted immediately to pH 5 to 6 add 2 ml. of the *reagent* and measure after 20 minutes the color value of the purple-red liquid. If less than 5 γ/ml. is present, use a larger amount of the solution. After 20 to 24 hours, the colored precipitate settles out and may be examined with a hand glass.

Having established the basic reaction of the method, Prebluda and McCollum, realizing the complications due to difference in composition of thiamin-containing foods, left special details to be elaborated by others.

Melnick and Field Method.[68] As applied to materials containing practically all the thiamin in the free state, this method, developed at the University of Michigan, employs a combination of the adsorption and elution procedures with the color reaction of Prebluda and McCollum. Yeast is subjected to hydrolysis by the phosphatase of dry yeast which liberates the free thiamin in phosphoric esters, including cocarboxylase, thus permitting the determination of total thiamin.

APPARATUS. *Adsorption Assembly* (Fig. 72). The parts are R, a graduated cylinder for the vitamin solution; T, a siphon tube with C_1, a stopcock leading through a double-bored rubber stopper into Q, a gradually tapering funnel-tube heated in the lower portion by J, a steam-jacket, and connected below with Z, a tube containing Permutit and at G glass-wool. The lower constricted end of the Permutit tube passes through a stopper into E, an Erlenmeyer flask provided near the mouth with C_2, a two-way stopcock; attached to the stopper are two glass hooks for suspending F, a receiving tube with a mark showing 10 ml. Through the other hole of the stopper closing the top of the funnel tube, passes a double-bent tube provided with C_3, a two-way stopcock which connects with W, a small filter flask containing cotton-wool and this in turn with N a gas bag containing nitrogen. A short test tube D with a 10-ml. graduated mark displaces R, the graduated cylinder, when acid potassium chloride is introduced into the funnel tube.

Micro Colorimeter.

REAGENTS. *Permutit.* A commercial zeolite. Treat by the Cerecedo and Kaszuba

process [69] to remove free alkali thus: Wash by suspension 5 times in water, 3 times in ethanol, once in acetone, and twice in anhydrous ether, then filter, dry at 50°, and store.

Prebluda and McCollum Reagent (Diazotized Amino-Acetophenone). See above.

PROCESS. A. FREE THIAMIN. *Extraction.* Weigh a quantity of finely divided rice polish, wheat germ, yeast, or other products, with low ratio of salts to vitamin, sufficient to yield an extract containing 150 γ of thiamin. Mix with 20 to 30 parts of water at 70° and adjust to pH 4.5. Heat for 30 minutes on a water bath at 70 to 72° under an atmosphere of *nitrogen*, stirring continually (preferably with a mechanical stirrer), centrifuge, and decant the liquid. Mix the residue with water and repeat the contrifuging, then combine the extracts and washings. If desired, extract with *80% methanol* for 10 minutes; it is quite as efficient as water.

After mixing liver powder and similar animal products with a minimum quantity of water, saturate the extract with *anhydrous sodium sulfate*, concentrate *in vacuo* to a sirup, then add *phenylcarbinol* and concentrate further until no aqueous phase remains. Prepare a water solution of the vitamin from the phenylcarbinol extract and washings as described above.

Adsorption. Pour 3 g. of the purified *zeolite* into the filter tube, with an inside diameter of 8 mm., on top of glass wool, thus securing a filtering column 10 cm. high. Since the same charge of Permutit is used several times after the potassium chloride elution and washing, previous to the first use, suck through it 30 ml. of *25% potassium chloride solution* (adjusted to pH 2 with hydrochloric acid) while passing steam through the jacket. Continuing the suction, wash with 500 ml. of hot distilled water. Stop the steam flow, open the filter chamber to the nitrogen reservoir, thus filling the system with the inert gas.

Siphon an aliquot of the vitamin solution (pH 4.5 and preferably non-buffered) from the measuring cylinder into the Permutit column. With the suction turned off and both two-way stopcocks open to the atmosphere, allow the percolation to proceed at room temperature by gravity at about 10 ml. per minute. When all the vitamin solution has been drawn into the siphon, add 30 ml. of water at pH 4.5 to the graduate and allow it to run into the siphon separated by a bubble of air from the vitamin solution until all the latter has passed into the filter column. Close stopcock C_1 and fill the graduate with wash water. Turn the two-way stopcock C_3 so as to permit the nitrogen gas to pass from the bag N into the funnel tube Q and apply suction to hasten the filtration, then open to the atmosphere as before, heating with steam, and allow the wash solution to flow into the filtering column.

Elution. Replace the graduate R by the test tube D containing 10 ml. of the *25% potassium chloride solution* and siphon into the funnel tube Q. At this stage, heat the wash solution in Q and draw it through the Permutit by suction with the stopcock C_1 open to the nitrogen bag N. This solution serves both to wash out the vitamin and to raise the temperature of the Permutit in Z to the optimum for elution.

Again open the system to the atmosphere, suspend the tube F by the glass hooks in E and siphon all the salt solution very slowly through the steam-jacketed tube, using at first only pressure from the nitrogen reservoir, then at the end suction, maintaining the temperature throughout at 65°.

Bring the solution caught in F up to the 10-ml. mark. After washing the Permutit with 500 ml. of hot distilled water, it is ready for the next analysis, the whole procedure being carried out in 20 minutes.

Neutralization. Pipet 3 ml. of the eluate into a 50-ml. centrifuge bottle, add 3 ml. of *ethanol* containing 5 mg. of *phenol* per milliliter and 1 drop of *thymol blue indicator*, then, while bubbling a fine stream of *nitrogen gas*

into the solution, add *1.0 N sodium hydroxide solution* dropwise until a faint but positive blue color appears.

Color Formation. Add to the blue liquid 6 ml. of *Prebluda and McCollum reagent,* stopper, and allow to stand overnight at room temperature.

Xylol Extraction. Shake the mixture vigorously with 2 ml. of *xylol* for 1.5 minutes. Only the color of the vitamin derivative is extracted by the xylol, the indicator color remaining in the aqueous phase.

Color Comparison. Compare the color of the xylol layer with that of 0.5 ml. of a standard solution containing 50 γ of thiamin chloride added to a 2.5-ml. aliquot of a 10-ml. blank potassium chloride eluate treated in like manner.

Time Element. One person in an eight-hour day can make 10 complete determinations with duplicate color comparisons of each.

B. Total Thiamin by Phosphatase Hydrolysis (Free Thiamin plus Phospho-Thiamins). Thiamin monophosphate and pyrophosphate (cocarboxylase) give orange and pink-colored derivatives with the Prebluda and McCollum reagent but, since these are not soluble in xylol, the color value of the xylol extract represents only the free thiamin. The phosphatase method is useful chiefly in determining the vitamin B_1 potency of yeast which contains as high as 75% of the phosphorylated vitamin. Yeast thus analyzed must not be confused with yeast the reagent, the vitamin content of which is determined by a blank analysis.

Hydrolysis. To 30 ml. of the extract containing about 100 γ of thiamin, prepared as directed under A, add 0.2 ml. of *toluene* and 0.5 g. of *yeast powder.* Adjust the suspension to pH 4.5 by adding *1.0 N sulfuric acid* or *sodium hydroxide solution* and incubate for 24 hours at 37.5°.

Color Formation and Reading. Filter the incubate and proceed as directed under A. Correct for thiamin present in the yeast powder, as determined by a blank analysis.

C. Thiamin as Phosphoric Esters (Cocarboxylase, etc.). Subtract the result for free thiamin (A) from that for total thiamin (B).

Note. Dr. Melnick states in a personal communication that a modification of this method by M. Hochberg and D. Melnick, which involves simplification of both apparatus and procedure, is in press.

Emmett, Peacock, and Brown Superfiltrol Modification.[70] The original Melnick and Field procedure in most details is followed in this modification developed in the laboratory of Parke Davis and Co., Detroit, but the complicated Permutit adsorption and elution are replaced by a simpler procedure with superfiltrol, and the Lovibond tintometer is substituted for the colorimeter.

Standard Curve. Prepare solutions containing 5, 10, 20, 30, 40, and 50 γ of crystalline thiamin per 3 ml., obtain the corresponding red values as in an actual analysis, and plot a standard curve showing gammas of thiamin as abscissas and red values as ordinates.

Process. *Extraction.* As in the original method above.

Dilution. Place in 15-ml. centrifuge tubes varying amounts (2, 3, 4 ml.) of a stock solution of the test material containing 5 to 20 γ/ml. of thiamin and dilute each to 5 ml. with water.

Adsorption. After adjusting to pH 4 to 5 with *dilute hydrochloric acid,* add 0.1 to 0.15 g. of *superfiltrol.* Let stand for 1 hour with occasional shaking, then centrifuge and discard the supernatant liquid.

When the vitamin is combined, as in dried yeast, it is necessary to split it off by digestion with a mixture of *taka-diastase* and *papain,* extraction, and adsorption from the extract with *superfiltrol.*

Color Formation. To the adsorbate, add 3 ml. of water and 3 ml. of *ethanol* containing 5

mg. of *phenol* per milliliter. After adding 1 drop of *thymol blue*, adjust to pH 7 to 8 with *dilute sodium hydroxide solution*, then add 6 ml. of the *Prebluda and McCollum reagent*, and thoroughly mix. Allow to stand for 2 hours or more at room temperature, filter on a small Hirsch filter, and wash the adsorbate with about 5 ml. of water.

Elution. Place the adsorbate and paper in a dry centrifuge tube, add 2 ml. of *ethanol*, stopper, and shake to dissolve the pigment. Centrifuge and decant into a tintometer cell.

Color Reading. Read in the Lovibond tintometer.

CALCULATION. Evaluate in terms of thiamin from the standard curve, taking the average of three results.

NOTE. Brown, Hartzler, Peacock, and Emmett [71] (Parke, Davis & Company, Detroit) obtained good agreement by the above procedure and the Hennessy and Cerecedo Thiochrome Method (below). Although the former is the more reliable, the latter, being more sensitive, is preferred for samples of low potency.

Meunier and Blancpain Sulfanilic-Thiochrome Colorimetric Method.[72] By combining the reactions involved in the Jansen thiochrome and the Kinnersley and Peters diazotized sulfanilic acid methods, Meunier and Blancpain evolved a method for the separate determination of both thiamin and cocarboxylase, as well as total thiamin, in dried yeast and yeast extracts. (Pasteur Institute, Paris.)

APPARATUS. *Electrophotometer* or *Nessler Tubes.*

REAGENTS. *Koesler and Hanke Diazotized Sulfanilic Acid Solution.* See Kinnersley and Peters Method above.

Modified Kinnersley and Peters Alkaline Reagent. Dissolve 2.86 g. of $NaHCO_3$ in 100 ml. of 0.5 N NaOH.

PROCESS. *Extraction.* See Jansen Method below and Kinnersley and Peters Method above.

A. FREE THIAMIN. *Color Formation.* Introduce into the colorimeter cell or Nessler tube 2 drops of *sulfanilic acid solution* and 4 ml. of a mixture of 6 volumes of freshly prepared *5% sodium nitrite solution* and 100 volumes of the *modified Kinnersley and Peters alkaline reagent.* Mix and, after 30 seconds, add 1 ml. of the extract of the sample, containing 3 to 30 γ of the vitamin, at pH 5 to 6.

Color Reading. Exactly 30 seconds after the last addition, make a preliminary reading with a green filter; then read after 15-second intervals up to 1.5 to 2 minutes.

Treatment after Oxidation. Oxidize another portion of the extract with 1 drop of *0.1% potassium ferricyanide solution* for each 5 ml. as directed under the Jansen Method below; then proceed with the color formation and reading as above.

The difference in the two readings represents free thiamin in the sample.

B. TOTAL THIAMIN. Liberate the thiamin by enzymic hydrolysis as directed under the Kinnersley and Peters or the Melnick and Field methods; then proceed as directed under A.

Marenzi Nitroaniline Colorimetric Method.[73] Instead of diazotized sulfanilic acid or diazotized amino acetophenone, used in the foregoing methods, Marenzi employs, for 10 γ or more of thiamin, *0.1% p-nitroaniline solution* in conjunction with 2 ml. of *16% sodium carbonate solution*, then heats 4 to 5 minutes in a boiling water bath. The red thiamin derivative is transferred to 2 ml. of *benzene* by shaking the yellow-brown precipitate in a separatory funnel.

Jansen Ferricyanide-Thiochrome Fluorimetric Method.[74] Peters [75] first observed that thiamin on oxidation in aqueous solution is converted into a substance characterized by its intense sky-blue fluorescence in ultra-violet light and suggested the relationship of the product to yellow fluorescent flavins and the Bence Jones "quinoidine" compounds in yeast extracts. Barger, Ber-

gel, and Todd,[76] by the action of *potassium ferricyanide* on thiamin hydrochloride in alkaline solution, obtained a pale yellow crystalline derivative with an intense blue fluorescence and other properties of thiochrome ($C_{12}H_{14}N_4OS$).

Jansen (Amsterdam University) applied the thiochrome reaction to the determination of thiamin. The Jansen method and its modifications have been extensively employed in vitamin assay. Hennessy and Cerecedo [77] have greatly extended the usefulness of the thiamin reaction by the use of a base-exchanging zeolite and the conversion of thiamin phosphoric esters, notably cocarboxylase, into thiamin, thus permitting the determination of the total (free and combined) thiamin. See below.

APPARATUS. *Cohen Fluorimeter* [78] or its equivalent. The instrument is of the objective type, the fluorescence being converted into electric current, the intensity of which is measured by a galvanometer. The fluorimeter employed by Hennessy and Cerecedo is supplied by Pfaltz and Bauer, New York City. As noted below, Karrer and Kubli find ordinary Nessler tubes adequate.

In standardizing the fluorimeter for each series of determinations, determine the fluorescence of a solution of 0.3 mg. of quinine (base) in 0.1 *N* sulfuric acid, as recommended by Kuhn and Vetter.[79]

PROCESS. *Extraction.* See details given by Pyke and by Hennessy and Cerecedo below.

Adsorption and Oxidation. If necessary, purify the solution by adsorption, following A or B.

(A) Place the solution, or an aliquot, containing 1 to 20 γ of thiamin hydrochloride in 0.1 ml. of water in a glass-stoppered 25-ml. graduated cylinder with *fuller's earth*, add a volume of *0.1% potassium ferricyanide solution* apportioned to the weight of thiamin as follows: 1 γ, 0.01 to 0.1 ml.; 10 γ, 0.03 to 0.1 ml.; 20 γ, 0.1 to 0.2 ml.

(B) For 10 to 50 mg. of *International Standard Adsorbate*, add *1% potassium ferricyanide solution* as follows: 10 mg., 0.05 to 0.10 ml.; 30 mg., 0.1 to 0.2 ml.; 50 mg., 0.3 ml. Stir the absorbate for 5 to 10 minutes to secure thorough mixture of the vitamin and fuller's earth vehicle.

In both (A) and (B) mix well with the ferricyanide solution and add 3 ml. of *10% sodium hydroxide solution.*

Thiochrome (but not thiamin) is stable in the mixture of ferricyanide and alkali.

Isobutanol Extraction. After 1 to 2 minutes, shake with 13 ml. of *isobutanol* and centrifuge.

Fluorescence Reading. Remove 10 ml. of the isobutanol extract to the cell of the fluorimeter and observe the deflection of the galvanometer needle.

NOTES. Otto and Rühmekorb [80] call attention to the necessity of avoiding long exposure of the thiochrome solution to ultraviolet light. In 5 minutes 20% is destroyed. Farrer [81] substitutes *butanol* for isobutanol for the extraction of thiochrome.

I. Pyke Modification.[82] Although contributing no novel features to the method proper, Pyke (London) gives useful instructions for extraction of the vitamin from various food products as follows. Grind or mince 25 g. of cereals or meat products, add 100 ml. of *1% hydrochloric acid*, stir vigorously, and bring just to boiling. Cool, make up to a convenient volume, and add three 3-ml. aliquots to a mixture of 2 ml. of *methanol*, 1 ml. of *30% sodium hydroxide solution*, and 0.8, 1.0, and 1.2 ml. of *1% potassium ferricyanide solution* respectively. Mix thoroughly, then shake out with 13 ml. of *isobutanol*, centrifuge, and proceed as above described.

Conduct a blank determination, omitting the addition of ferricyanide, and introduce the correction. If the three results on the unknown do not agree closely, a different range of potassium ferricyanide must be tried until the maximum fluorescence is obtained.

Boil 5 g. of finely ground wheat germ or disintegrated yeast with 100 ml. of *1% hydrochloric acid* and proceed as above. Add the *reagent* directly to 5 ml. of milk.

NOTE. McFarlane and Chapman[83] destroy interfering pigments by the addition of *hydrogen peroxide* to the blank and the unknown before extracting with *isobutanol*. The readings are made in the Froman and McFarlane fluorimeter.[84]

II. Karrer and Kubli Modification.[85] Somewhat more *ferricyanide solution* is used in this modification than in the original Jansen method; it further differs in that at least 0.4 ml. for 20 mg. of *fuller's earth standard* is essential and 2.0 ml. is without detrimental influence. Instead of measuring the fluorescence with the fluorometer, they compare visually the fluorescence of the isobutanol extract with that of an isobutanol extract of a standard solution of approximately the same vitamin content, both mixed with 25 ml. of water, 0.05 ml. of *1% potassium ferricyanide solution* and 3 ml. of *10% sodium hydroxide solution*. The known and the unknown are irradiated horizontally and vertically with quartz lamps for 1 to 1.5 minutes. A stock solution containing 5 I.U. per milliliter keeps well in a refrigerator.

NOTE. Vastagh[86] follows in essential details the Karrer and Kubli modification, but employs larger quantities of solution and reagents and substitutes for direct observation reading in the Pulfrich photometer in conjunction with the Hanau analyzing lamp. His best results were obtained when the proportions used were 5 γ of thiamin hydrochloride in 10 ml. of water, diluted to 150 ml., 5 ml. of *1% potassium ferricyanide*, 10 ml. of *10% sodium hydroxide solution*, 20 ml. of *isobutanol*, and, if the extract is opalescent, 1 g. of *anhydrous sodium sulfate*.

III. Mukherji Modification.[87] In the determination of thiamin in cereals, vegetables, and commercial concentrates, Mukherji oxidizes the vitamin in an atmosphere of *carbon dioxide*, extracts with 3 to 4 ml. instead of 13 ml. of *isobutanol*, thus increasing the fluorescence, and measures the fluorescence in the Pulfrich photometer.

IV. von Mallinckrodt-Haupt Modification.[88] The fluorescence is compared in the Pulfrich photometer with standard fluorescent glass. By adding 2 ml. of *acetic acid* to 4 ml. of the isobutanol extract, a green instead of a blue fluorescence is obtained which is stated to increase the range that can be read in the instrument from 2 to 250 γ. Otto and Rühmekorb[89] criticize the modification.

V. Kavanagh Modification.[90] The details were developed in connection with studies made at the New York Botanical Garden.

APPARATUS. *Klett Fluorimeter.*

Leeds and Northrup Type R Galvanometer.

REAGENTS. *Butanol*, 1.0 *N*.

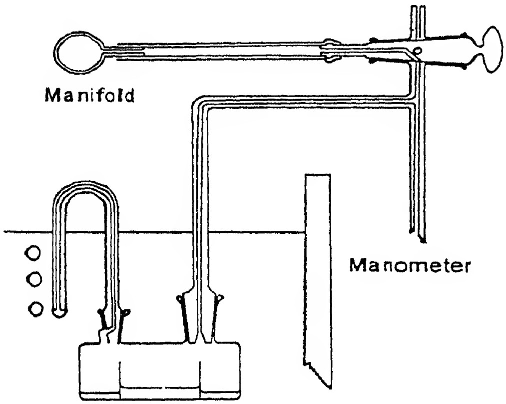
Other reagents as given above.

PROCESS. Thiochrome decomposes rapidly when irradiated by 3660 Å lines and is about half destroyed in 10 minutes. Measurements are made as follows.

Place in position the quinine standard, also lamp filter for the standard 597 (5.25 mm.) and the photocell filter 306 (3.2 mm.), then fill the cell with the thiochrome solution of the unknown. Start the stopwatch the moment the cell enters the ultra-violet light beam. Measure the fluorescence, at least 4 times in the first 2 minutes, correct for the fluorescence of a blank, and plot the logarithm of the corrected potentiometer readings against time. To obtain the potentiometric reading of the undecomposed thiochrome, extrapolate to zero time. Also plot these readings at zero time for solutions of different concentrations against the concentration, thus obtaining a straight line. If results with 1 to 2% of the truth are sufficiently accurate, read the potentiometer immediately and assume that no considerable decomposition has taken place. The limits for the thiochrome method in the instrument are between 150 and 1 γ per liter.

Butanol removes grease from the stopcocks; Whatman filter paper No. 42 yields fluorescent substances.

Schultz, Atkin, and Frey Yeast Fermentation Method. The discovery by Schultz, Atkin, and Frey (Fleischmann Laboratories, New York)[91] that thiamin acts as a powerful stimulant of alcoholic fermentation by yeast led to the application of the principle in a method depending on the amount of carbon



Courtesy of the Authors and J. Biol. Chem. 1939, **129**, 472

FIG. 73. Schultz, Atkin, and Frey (Warburg) Thiamin Apparatus.

dioxide gas evolved in a given time and utilizing the assembly of thermostat, shaker, and gasometer devised by Schultz and Landis.[92]

WARBURG APPARATUS. The conventional type except for a special flask (Fig. 73), maintained at 30°, with an outside diameter of 60 mm. and a capacity of about 70 ml. An inset cup for holding the reaction mixture (yeast, sugar, etc.) has a diameter of 25 mm. This cup is surrounded in the main chamber by 1 ml. of water to aid in temperature equilibrium. A capillary gas valve vents beneath the surface of the water, thus permitting observation of escaping gas during the passage of nitrogen which is maintained at constant, low pressure by means of a simple pressure regulator set at 20 cm. of water.

REAGENTS. *Sugar and Salts Solution.* Dissolve in water and make up to 1 liter 200 g. of c.p. dextrose, 2.2 g. of KH_2PO_4, 1.7 g. of $CaCl_2 \cdot 2H_2O$, 10 g. of $MgSO_4 \cdot 7H_2O$, 0.067 g. of nicotinic acid, 0.01 g. of $FeCl_3 \cdot 6H_2O$, and 0.01 g. of $MnSO_4 \cdot 4H_2O$.

Nicotinic acid is included in the reagent to render impotent that present in the material analyzed, since, according to Schultz, Atkin, and Frey,[93] it has slight vitamin B action, reaching a maximum at 4 γ.

Citrate-Phosphate Buffer. Dissolve 70 g. of $H_3C_6H_5O_7 \cdot H_2O$ and 119 g. of $K_2HPO_4 \cdot 3H_2O$ in water and make up to 1 liter.

Ammonium Sulfate Solution. Dissolve 150 g. of $(NH_4)_2SO_4$ in water and make up to 1 liter.

Yeast Suspension. Prepare a suspension of 2 g. of commercial baker's yeast (Fleischmann) in 100 ml. of water.

Gelatin Solution. Dissolve 1 g. in 100 ml. of water.

Thiamin Stock Solution. Dissolve 1 mg. of Merck's synthetic thiamin chloride hydrochloride in water and make up to 1 liter; 1 ml. = 1 γ thiamin.

Thiamin Standard Solution. To 1 ml. of gelatin solution in a 100-ml. volumetric flask, add about 25 ml. of water and shake well to wet the whole interior, then add 2 ml. of the thiamin stock solution (2 γ) and make up to volume. Prepare daily.

Sterilize other solutions in cotton-plugged Erlenmeyer flasks on successive days for 30 minutes in flowing steam. When a flask has been opened, keep in a refrigerator.

PROCESS. *Sample.* Grind if necessary cereals, oil seeds, legumes, and other seeds or their products, suspend in water, acidify to *Congo red*, heat at 100° for 30 minutes, cool, and make up to volume with water, then pipet an aliquot directly into the Warburg flask. Simply heat milk in an Arnold steam sterilizer and dilute with water.

Fermentation. Grease all ground joints with *anhydrous lanolin*, open the capillary gas

valves, and start the flow of *nitrogen* through the train. Place 1 ml. of water in the main chamber of each flask and add to the inset cup 1 to x ml. of water followed by x ml. of the solution of the unknown or the thiamin standard. When all is in readiness, place in a 100-ml. flask 15 ml. of the *sugar and salts solution*, 10 ml. of *buffer*, and 7 ml. of *ammonium sulfate solution*, then add 25 ml. of *yeast suspension*, start a timer, and make up to volume with water. Add 1 ml. of this mixture to the inset cup of each flask, immediately connect with the manometer, place the whole in a thermostat, and flush with *nitrogen*. Usually 10 to 12 minutes will have elapsed in performing these operations. Shake the flasks at 120 oscillations per minute at an amplitude of 3 cm. for 40 minutes, continuing the flow of *nitrogen*.

Reading. When the total elapsed time reaches about 55 minutes, release the excess pressure and at exactly 60 minutes take an initial or zero reading. After 1 hour, take a final reading.

Calculation. Calculate the fermentation rate per hour. The relation between stimulation and quantity of thiamin is approximately linear up to 30 mγ at which point the stimulation is 380 c.mm. Therefore in practice determine a portion of a curve in each run. Measure the gas produced by 10 to 20 γ of pure thiamin and estimate aliquots of the unknown, containing intermediate quantities of thiamin, on the basis of linear relationship.

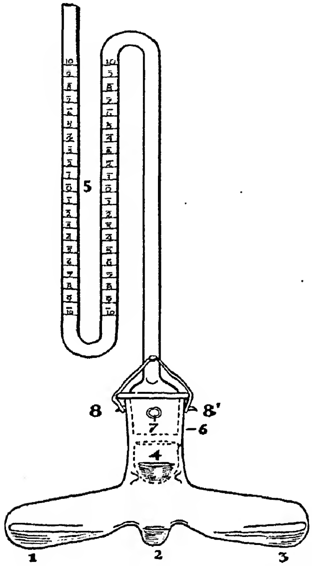
Examples. The data given in the following table illustrates the application of the method:

Addition to Control Reaction Mixture	Gas Produced	Thiamin Found	Thiamin: Ultra-micro method	Thiamin: Original gas method
	c.mm. per h.	mγ	γ/g.	γ/g.
0.5 mg. rice polish A	588	10.4	20.8	22.0
0.02 mg. dry yeast A	686	18.1	904.0	960.0
0.02 mg. dry yeast B	624	13.2	66.0	68.0
0.035 ml. milk, grade B	635	14.1	0.40	0.40
10 mγ thiamin	583	....	....	....
20 mγ thiamin	710	....	....	....

I. Modification for Differentiating Thiamin from Derivatives.[94] To 35 ml. of a solution of the unknown, add 25 ml. each of *1% potassium ferricyanide solution* and *50% sodium hydroxide solution*, then allow to react at room temperature for 5 minutes, during which time the thiamin will be completely oxidized, forming thiochrome which has no stimulating action. Neutralize with *dilute sulfuric acid*, make up to 100 ml., and employ an aliquot of 25 ml. in the fermentation procedure as described above.

II. Sulfite Modification. Schultz, Atkin, Frey, and Williams[95] found that in applying the method to materials of low vitamin potency, such as refined white flour, extraction and concentration of the vitamin are necessary. They have also devised a modification based on the cleavage of the thiamin by sodium sulfite into 2-methyl-6-amino-pyrimidine-5-methyl-sulphonic acid and 4-methyl-5-(β-hydroxy)-ethylthiazole, neither of which

is active in yeast fermentation. 2-Methyl-5-ethoxymethyl-6-aminopyrimidine, a possible interfering substance in the original method, and the corresponding 5-hydroxymethylpyrimidine and its esters, also classed as interfering substances, are not affected by the sulfite treatment.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1942, **14**, *279*

FIG. 74. Bunzell Reaction and Manometer Assembly.

PROCESS. To 20 ml. of the solution or suspension of the unknown, add 0.2 g. of *sodium sulfite* ($Na_2SO_3 \cdot 7H_2O$) and adjust to *p*H 5. Heat in flowing steam for 30 minutes, cool, and destroy the excess of sulfite with *3% hydrogen peroxide*, using *starch-iodide solution* on a spot-plate as indicator. Adjust to *p*H 6.2, make up to a convenient volume, and subject an aliquot to the fermentation procedure as described above.

III. Schultz, Atkin, and Frey Fermentometer Modification.[96] Sulfite cleavage and a new multiple gasometer are featured.

IV. Bunzell Simplified Modification.[97] APPARATUS. *Reaction and Manometer Assembly* (Fig. 74). The parts are (1) compartment containing 1 ml. of nutrient solution[91] and 1 ml. of vitamin-containing extract or standard thiamin solution, (2) compartment used only when the effect of other substances on the reaction is studied, (3) compartment containing 1 ml. of 0.4% yeast suspension (free from added vitamins), (4) not used for present purpose, (5) manometer graduated so each division is equivalent to 100 γ of carbon dioxide when the apparatus is charged, (6) ground joint with corresponding vent holes, (7) pressure equalizer for use when manometer is at right angles to the body of the apparatus, and (8 and 8′) rubber bands holding parts in place. The figure shows the manometer filled with mercury closed by rotating through 90°.

Constant Temperature Chamber.

PROCESS. Run concurrently knowns and unknowns, with and without sulfite. Clamp the apparatus on a shaking machine mounted in a constant temperature chamber (shown in the article) maintained at 30°. Adjust the air vents to allow for equalization of internal and external pressure. After 25 minutes, close the apparatus as described, using a special trapdoor in the chamber.

Readings. Start the shaking machine and take readings after 90, 120, 150, and 180 minutes. Interpret the readings of the unknown in terms of B_1 concentration by interpolating from readings of standards. The Bunzell shaking machine has a stroke of 6 cm. and a period of 2 excursions per second.

Heyns Yeast Fermentation Method.[98] The method (E. Merck, Darmstadt) is based on the assumption that thiamin and cocarboxylase (thiamin pyrophosphate) yield carbon dioxide in equal molecular proportions.

APPARATUS. *Reaction Flasks,* of 200-ml. capacity, each with a double-bored rubber stopper, through one hole of which passes a double-bent glass tube provided with a stop-

cock, through the other hole a short tube extending only a few millimeters within the flask. A mechanical shaker, holding 6 reaction flasks, is submerged in a water bath held at 30°.

Hayduck Gas Burets, 1-liter capacity not including leveling bulb, diameter 4 cm., length 115 to 120 cm., each closed at the top by a double-bored rubber stopper, through one hole of which passes a glass tube provided with a stopcock, through the other hole, a short tube connected by a rubber tube with the reaction flask.

Reagents. *Buffer Solution A.* Dissolve 50 g. of monoammonium phosphate and 30 g. of diammonium phosphate in water and make up to 1 liter.

Nutritive Salts Solution B. Dissolve in water 350 g. of dextrose, 20 g. of monopotassium phosphate, 5 g. of dihydrated calcium chloride ($CaCl_2 \cdot 2H_2O$), 10 g. of crystalline magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 5 mg. of manganese sulfate, and 5 mg. of ferric chloride, and make up to 1 liter.

Yeast Suspension. Thoroughly mix 10 g. of finely divided compressed yeast with 200 ml. of water.

Process. *Extraction.* See foregoing methods.

Incubation. Introduce into each reaction flask 15 ml. of *Solution A* and 25 ml. of *Solution B*, dilute with 40 ml. of liquid (40 ml. of water for a blank, 40 ml. of extract for the actual determination, and 40 ml. of water containing 1, 5, 10, and 25 γ or other suitable amounts of *thiamin hydrochloride* for standards).

Close each flask, place in the shaker, submerge in the bath, connect with a buret previously filled to the mark with water colored with *dinitrophenol* and covered by a layer of *mineral oil*, and displace the air in the system by means of a current of *carbon dioxide;* then add 20 ml. of the *yeast suspension* and adjust the liquid in the buret to a suitable initial volume by the leveling bulb.

Reading. At intervals read the volume of carbon dioxide evolved during fermentation. The most satisfactory results are obtained usually after 6 hours.

Calculation. Introduce the correction for the blank and estimate the amount of thiamin by comparison with the standards. If the unknown is outside the range of the standards or if the gaps in the standards are too wide, repeat the incubation, using solutions of suitable dilution.

Schopfer Fungus Growth Gravimetric Method. Schopfer [99] proposed this delicate method for vitamin B_1, using *Phycomyces Blakesleeanus*, a fungus of the family *Mucoraceæ*, which is exceedingly sensitive to the vitamin. The distinctive feature of the method is the weighing of the dried mycelium of the fungus, representing the growth on a culture medium at *p*H 5, as distinguished from yeast methods where the generated carbon dioxide is measured. Later Schopfer and Müller [100] found it desirable to adsorb the vitamin on fuller's earth and elute before applying the method.

Williams [101] in 1937 warned against the use of fungi in quantitative work; Meiklejohn,[102] however, modified the method so as to make it a simple test for small samples of blood. Villela [103] applies the method to maté. Paleĭ [104] bases a method on the growth of the fungus.

I. Meiklejohn Modification.[102] Whether or not Meiklejohn's modification corrects the defects of the original method, the steps in the process are clearly described and are here given in sufficient detail for practical use. Although devised for blood only, the principle has been applied in the analysis of a variety of foods.

Reagents. *Culture Media.* (*1*) *For Blood.* Dissolve in water 10 g. of dextrose, 0.2 g. of asparagine, 0.05 g. of $MgSO_4 \cdot 7H_2O$, and 0.15 g. of KH_2PO_4 and make up to 100 ml.

(*2*) *For Standard.* Use the same quanti-

ties of dextrose and salts as above, but double the amount of asparagine, and add 0.3 ml. of 0.1 *N* NaOH per 10 ml. to bring the *p*H to 6.7.

Inoculation Medium. Dissolve 25 ml. of malt extract and 4 g. of agar in distilled water and make up to 200 ml.

Standard Thiamin Solution. Prepare a concentrated stock solution of known strength and *p*H 4.5. Keep in a refrigerator.

Spore Suspension. Two weeks before needed, prepare as follows. Sterilize in a 200-ml. conical flask about 70 ml. of the inoculation medium in an autoclave at 110° for 15 minutes and inoculate with about 0.5 ml. of a suspension of the fungus *Phycomyces Blakesleeanus* (sex). After 2 weeks, remove with sterile forceps several grams of the spore-bearing mycelium and wash thoroughly in 10 to 20 ml. of sterile distilled water contained in a beaker covered with a watch glass. Remove the mycelium, leaving a suspension of spores in the water (at least 1,000,000 per milliliter; check if necessary in a blood-counting chamber).

PROCESS. *Preparation of Blood.* Into each of a series of 50-ml. Erlenmeyer flasks with a mark at 10 ml., measure 6 ml. of the *culture medium.* To two of these add 2 ml. of the blood, collected in the presence of 3 mg. of *potassium oxalate* for each milliliter of blood. It is also desirable to add to another flask 1 ml. and to two other flasks 3 ml. of the blood sample. To two additional flasks, add 2 ml. of the blood together with measured portions of the *standard solution containing 0.1 γ of thiamin.* Store the four (or seven) flasks at −2° for 12 hours or longer to allow the corpuscles to settle out. Adjust the surface layer to *p*H 6.5 to 6.7 with 0.1 to 0.2 ml. of *0.1 N sodium hydroxide solution,* and dilute to the 10-ml. mark with water.

Preparation of Standard. To each of a second series of 50-ml. Erlenmeyer flasks, add 10 ml. of culture medium 2 and carefully measured volumes of thiamin solution (1 γ/ml.) freshly prepared from the concentrated standard solution. The usual quantities of vitamin selected for the range are 0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 γ, those of even tenths being in duplicate.

Sterilization. Autoclave both series of flasks at 107° for 10 minutes.

Inoculation. To each flask, add from a sterile pipet 0.2 ml. of the *spore suspension* and stir to prevent sedimenting.

Weighing. After 10 days in the dark room at room temperature, when all growth should have ceased, remove the mat of mycelium, wash under the tap, dry on a watch glass at 110° in an air oven to constant weight, and record the weight to the nearest 0.5 mg.

CALCULATION. Plot the dry weights of the mycelium obtained against the amount of crystalline thiamin added.

II. Sinclair Modification.[105] Sinclair (Oxford University) examined the Meiklejohn modification in great detail and concluded that it is neither accurate nor specific for vitamin B_1, although sensitive for assaying pure solutions of the vitamin. He intimates that if the limitations of the method are recognized, it may have value in comparing the apparent vitamin content of blood samples. His procedure is as follows.

REAGENTS. *Culture Media.* See above. Bring to *p*H 6.5 with 10 *N* NaOH and make up so that 4 ml. will be equivalent to the 6 ml. used by Meiklejohn for each 50-ml. flask. A better source of nitrogen than 0.4% asparagine is *hydrolized casein* prepared as follows: Heat 100 g. of caseinogen (Glaxo) with 300 ml. of HCl and 300 ml. of water under a reflux condenser for 3.5 hours, bring to *p*H 6.5 with NaOH solution, and make up to 950 ml.

Magnesium Sulfate Solution. Dissolve $MgSO_4 \cdot 7H_2O$ in water and make up to 0.002 *M.*

Spore Suspension. Prepare so that 1 ml. contains 15 million spores.

PROCESS. Add the blood to the medium, allow to stand at least 30 minutes, then add water and allow to stand at −2° for at least 12 hours before sterilizing by steam for 20 minutes on 3 successive days. Use cultures grown in a dark room for 10 days at a constant temperature of 18°.

FREE AND COMBINED THIAMIN

(Thiamin and Cocarboxylase)

Westenbrink and Jansen Thiochrome Fluorometric Method.[106] The method differs from the Jansen method chiefly in that in addition to determining the free thiamin by the fluorescence of the isobutanol layer, these authors determine the thiamin pyrophosphate (cocarboxylase) by the fluorescence of the aqueous layer. Previous to the oxidation, they adsorb the vitamin on *frankonite* (a silicate similar in absorptive properties to fuller's earth) and elute with *methanol* and *30% sodium hydroxide.*

Hennessy and Cerecedo Clarase-Thiochrome Fluorometric Method.[107] Three interrelated laboratory methods and one calculation method, developed at Fordham University, are described in one paper.

I. Hennessy and Cerecedo Modification of the Jansen Direct Thiochrome Fluorometric Method.[107] This method is used when the blank is small and recovery of added thiamin is quantitative.

APPARATUS. *Pfaltz and Bauer Photoelectric Fluorometer. Calibration.* Determine with wide-open iris diaphragm in the path of the incident ultra-violet light the galvanometer deflection (measuring the fluorescence) respectively for standard quinine sulfate solution in 0.1 N sulfuric acid (Q), for a thiochrome solution prepared by procedure (a) from N micrograms of thiamin chloride (T), and for isobutanol alone (B). With these data and Q_0 representing the quinine deflection for a lower incident intensity, calculate the deflection per microgram of thiamin chloride (D) by the following formula:

$$D = Q_0 \frac{T - B}{NQ}$$

Plot the deflections of the galvanometer against various readings of Q_0 to set the deflection per milligram of thiamin chloride.

In the measurement of the unknown, divide the difference in the deflections induced by oxidized and unoxidized aliquots by the deflection per microgram of thiamin chloride as set directly by reference to Q_0 or indirectly to the rear photocell deflection immediately before pouring the thiochrome solution into the cell.

PROCESS. *Extraction.* (*a*) Reflux for 3 minutes a weighed portion of the finely divided sample of cereals and cereal products, such as dried germ meal, with 5 to 20 parts of *2% acetic acid.* Cool, centrifuge, and filter. Repeat the operation and combine the extracts. To remove the emulsion formed with gelatin, add *ethanol* to a content of 30%. For use under methods II and III, remove the ethanol by vacuum distillation, replacing the loss with water. If, as is usual, the extract does not represent the total volume of acetic acid added, introduce a suitable correction. Precipitate proteins from milk and animal products with *2% trichloroacetic acid*, avoiding an excess, reflux, cool, and centrifuge.

(*b*) Extract 100 mg. of adsorbates, such as International Standard or U.S.P. Reference Standard, with four 5-ml. portions of boiling *2 N hydrochloric acid*, separating the solid matter by centrifuging. Extract the residue twice by mixing with 10 ml. of cold *2 N sodium hydroxide solution*. Combine the acid and alkaline eluates, adjust to the required pH, and make up to 50 ml. in a volumetric flask.

(*c*) Dilute water-soluble concentrates to the desired volume.

Oxidation. Make up a volume of the solution containing 0.2 to 20.0 γ of thiamin to 5

ml. in a 25-ml. glass-stoppered graduated cylinder. Add 0.05 to 0.20 ml. of *1% potassium ferricyanide solution* and 3 ml. of *15% sodium hydroxide solution,* then mix.

Isobutanol Extraction. Add immediately 13 ml. of *isobutanol* to the alkaline liquid, shake vigorously for 1 minute, and centrifuge for 30 seconds, then pour into a separatory funnel, remove the lower layer, and set aside for later comparison with the blank to learn if the procedure according to method III is necessary. Transfer the isobutanol solution to a test tube, add 2 to 4 g. of *anhydrous sodium sulfate,* mix well, and allow to settle.

Fluorescence Reading. Decant 10 ml. of the isobutanol solution, which must be perfectly clear, to the cell of the fluorometer and take the reading. Also read the fluorescence of an isobutanol solution obtained in a blank determination carried out in the same manner as the actual analysis, except that potassium ferricyanide is omitted and the order of the addition of sodium hydroxide solution and isobutanol is reversed.

II. Hennessy and Cerecedo Decalso Exchange Thiochrome Fluorometric Method.[107] For use when method I is not practicable, though no unusual unextractable fluorescence is noted.

Apparatus. *Exchange Tube.* The tube holding the adsorption column of Decalso has an inside diameter of 7 mm. and is provided with a funnel top and a two-way stopcock at the bottom for gravity flow and suction. The lower end is constricted to hold a plug of glass wool, above which is the 20-mm. column of 30-mesh Decalso. The tube is jacketed for 40 cm. of its length.

After introducing the Decalso, alternately pass four 15-ml. portions of boiling *2% acetic acid* and *25% potassium chloride solution* through the tube, keeping the temperature as near 100° as possible by passing steam through the jacket. Finally wash the column with three 15-ml. portions of boiling water. By this treatment excess of alkali is removed and conversion into the potassium salt is assured.

After each determination, particularly if the vitamin content is high, wash thoroughly with hot water, thus preparing the tube for subsequent determinations. Refill when the results on pure thiamin show either a large blank or a low recovery.

Process. *Extraction.* See method I.

Exchange Procedure. Pipet 5 to 20 ml. of the solution containing, as shown by the rough direct assay, up to 10 γ of thiamin and adjust to pH 4.0 to 4.5 with *acetic acid.* Bring to a boil and pour into the exchange tube which is heated to nearly 100°. Allow the solution to pass through the *Decalso* in 3 to 5 minutes, then wash with three 5-ml. portions of boiling water. Turn the stopcock to suction to remove the excess of water. Introduce boiling *25% potassium chloride solution* through the funnel top and regulate the flow to about 1 ml. per minute, collecting 10 to 25 ml. according to the vitamin content thus: 0 to 1 γ, 10 ml.; 1 to 3 γ, 15 ml.; 3 to 7 γ, 20 ml.; and 7 to 10 γ, 25 ml. After experience, the amount may be judged by the appearance of the oxidized solution in ultraviolet light.

Oxidation and Fluorescence Reading. Proceed with 5 ml. of the potassium chloride eluate as in method I, using 0.05 ml. of *1% ferricyanide solution.*

III. Hennessy and Cerecedo Enzymic Hydrolysis Thiochrome Fluorometric Method.[107] For use when considerable fluorescence remains in the alkaline layer.

Reagent. *Powdered Enzyme Concentrate.* Extract defatted beef kidney by shaking with an equal weight of 1% sodium chloride solution for 2 hours, centrifuge, and set aside in the refrigerator; reextract the residue in the same manner. Stir the combined extracts with sufficient 300-mesh Volclay (supplied by American Colloid Co., Chicago) to make a thin paste and centrifuge. Filter, add to the clear extract one-half its volume

of acetone, and centrifuge again. Add a second portion of acetone of the same volume as the first and stir. Collect the precipitate by centrifuging, dry in a vacuum desiccator over sulfuric acid, and powder.

PROCESS. *Extraction.* See method I.

Hydrolysis. Adjust the solution to pH 6.5 to 7.0, mix 5 to 20 ml. with 20 to 80 mg. of the *powdered enzyme concentrate*, and keep at 37° for 3 hours. Acidulate by adding 1 drop of *acetic acid*, boil, cool, and centrifuge.

Oxidation and Fluorescence Reading. Proceed with an aliquot of the centrifugate as in method I or, if interfering substances are present, as in method II.

IV. Calculation Method for Thiamin as Phosphoric Ester. Subtract from the result by method III the result by method I or II.

Conner and Straub Modification.[108] After systematic studies of the steps in the original process and its modifications, Conner and Straub (General Foods Corporation Central Laboratories) introduced the following improvements: (1) extraction and hydrolysis are conducted in the same vessel, (2) cocarboxylase is hydrolyzed by the enzyme clarase, and (3) the amount of sodium hydroxide is carefully adjusted.

APPARATUS. *Combined Extraction and Hydrolysis Apparatus* (Fig. 75). The capacity of the flask is about 75 ml. The stirrer (C) is operated by a small motor.

Decalso Absorption Tubes.

Incubator.

Pfaltz & Bauer Flurophotometer.

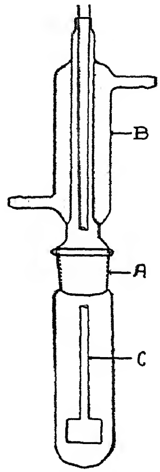
REAGENTS. *Clarase Solution*, 5%, in sodium acetate-acetic acid buffer solution (pH 4.5), prepared by dissolving 66.938 g. of anhydrous $NaC_2H_3O_2$ in water, adding 54.40 ml. of glacial $HC_2H_3O_2$, and diluting to 1 liter. Make up a fresh supply daily.

Isobutanol. Test the fluorescence of each lot. If greater than for distilled water, redistil in all-glass apparatus, collecting the 106 to 107° fraction.

Alkaline Potassium Ferricyanide Solution. Dissolve 0.2 g. of $K_3Fe(CN)_6$ and 45 g. of NaOH in water and dilute to 100 ml.

Other reagents as given for the Hennessy and Cerecedo method.

PROCESS. *Extraction.* Place in the extraction tube (Fig. 75) 3 to 5 g. of the finely pulverized sample (fresh vegetables are frozen with *solid carbon dioxide* and ground in



Courtesy of Ind. Eng. Chem., Anal. Ed. 1941, **13**, 380

FIG. 75. Conner and Straub Thiamin Extraction and Hydrolysis Assembly.

a food chopper and samples of high oil content are defatted), add 50 ml. of *0.04 N sulfuric acid* and, before heat is applied, stir thoroughly with the glass stirrer (C) to prevent subsequent charring. Boil over a micro burner or on a hot plate or else heat in a boiling water bath for 1 hour, with continuous stirring, then rinse the stirrer with 5 ml. of water, and cool under the tap to room temperature.

Incubation. Add to the mixture 10 ml. of *5% clarase solution*, stir with a glass rod, and place the tube with rod in an incubator at once, where it remains for 2 hours with frequent stirring. After the incubation, rinse

the stirrer with 1 ml. of water delivered from a pipet, cool to room temperature, and centrifuge at high speed until the supernatant liquid is clear.

Absorption by Decalso. Pipet an aliquot, containing about 5 γ of thiamin, into a 50-ml. beaker containing 5 ml. of *2% acetic acid* and heat just to boiling on an electric plate, then pass through a 5-cm. column of 60- to 80-mesh *specially activated Decalso* at the rate of 1 ml. per minute, using gentle suction for a moment when the solution reaches the bottom. The Decalso rests on a plug of spun glass in a tube similar to that employed for the fluorometric method described by Merck & Co. After the solution has passed through, rinse the beaker several times with hot water, and pass the washings through the column. The absorption of the vitamin is practically 100%.

Elute the vitamin from the column with 25 ml. of hot *25% potassium chloride solution*, following the directions of Hennessy and Cerecedo, and pipet 5 ml. of this eluate into a separatory funnel with a No-lub stopcock. To the aliquot, add 1 ml. of freshly prepared *alkaline potassium ferricyanide solution*, followed by 20 ml. of *isobutanol*, shake for 1 minute in a mechanical shaker, and centrifuge. Draw off the lower aqueous layer and treat the isobutanol layer with 2 g. of *anhydrous sodium sulfate*, then again centrifuge. Perform both oxidation and extraction as rapidly as possible.

Fluorescence Measurement. Pipet 15 ml., or other suitable aliquot, into the cell of the fluorophotometer and proceed as directed by Hennessy and Cerecedo above.

Check the instrument before each measurement with a standard quinine sulfate solution.

EXAMPLES. Barley 4.86 to 5.28, wheat 1.05 to 6.87, wheat germ 34.68, fortified flour 1.56, fresh Lima beans 2.97, fresh broccoli 1.26, fresh string beans 0.93, fresh squash 1.47, frozen peas 1.37 to 4.32, whey powder 1.69, skim milk powder 3.87, cocoa 0.84, and green coffee 2.10 and yeast 187.0 γ/g.

THIAMIN AND RIBOFLAVIN

Conner and Straub Combined Decalso-Supersorb Fluorometric Method.[109] The separation of thiamin from riboflavin depends on the absorption of the former by Decalso and of the latter by Supersorb.

APPARATUS. *Multiple Absorption Assembly* (Fig. 76). The parts (manufactured by the Scientific Glass Apparatus Co., Bloomfield, N. J.) are (1) two superimposed tubes, like the one used for the determination of thiamin alone by the Conner and Straub Modification of the Hennessy and Cerecedo Method (above), the upper one being for Decalso, the lower one for Supersorb, and (2) a graduated vacuum chamber with cocks as shown. Ferrebee[110] employs a longer (15 cm.) tube for Supersorb, since the material is only ground to pass a 30 to 60 mesh.

Eastman Safety Lights, equipped with series OA Wrattan light filters.

Pfaltz and Bauer Fluorophotometer.

PROCESS. The whole procedure must be carried out under controlled illumination such as that obtained with Eastman safety lights equipped with series OA Wrattan light filters.

Extraction and Incubation. Proceed as directed for the Conner and Straub modification of the Hennessy and Cerecedo method.

Absorption. Place in the upper tube a 5-cm. column of 60- to 80-mesh *Decalso* and in the lower tube a 5-cm. column of 60- to 80-mesh *Supersorb*. Pipet an aliquot containing about 5 γ of thiamin into the upper tube. This same aliquot also serves for the determination of riboflavin, if the amount of the latter does not vary greatly from 1 γ; otherwise a separate aliquot must be used for that determination.

Pass the aliquot through the two columns at the rate of 1 ml. per minute, regulating the

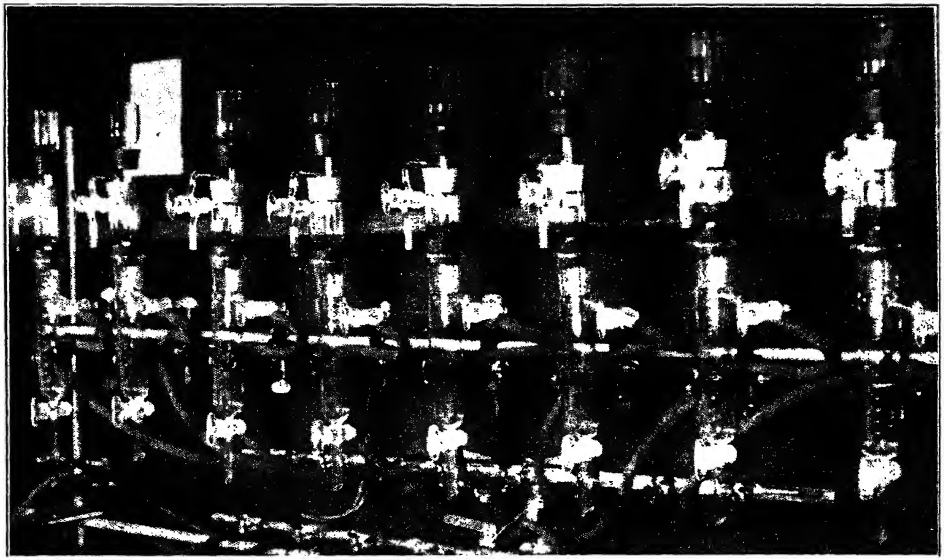


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FIG. 76. Conner and Straub Multiple Thiamin Adsorption Assembly.

flow by the stopcock between the two columns and that in the vacuum receiver, the latter being lubricated with *glycerol* or *orthophosphoric acid* to avoid the fluorescent constituents of other lubricants. Wash both columns with hot water and discard.

A. THIAMIN. *Elution.* Transfer the upper tube to a 125-ml. suction flask, elute the thiamin with 25 ml. of hot *25% potassium chloride solution* and proceed as directed for the Conner and Straub modification of the Hennessy and Cerecedo method.

B. RIBOFLAVIN. Detach the lower tube, empty the receiver, wash with hot water, and dry in an air current, then replace the tube and elute the riboflavin with 25 ml. of *20% pyridine solution in 2% acetic acid* (stored in the dark), added from an automatic pipet at the rate of 1 ml. per minute. Continue the addition of the eluant until the volume indicated in the receiver totals 50 ml., then make up to exactly 50 ml. (or for concentrates to a larger volume) in a volumetric flask with the eluant.

Color Formation. Remove an aliquot of 15 ml., containing 0.5 to 1.0 γ of the vitamin, to a 50-ml. brown bottle, add 1 ml. of *4% permanganate solution*, followed a minute later by 3 ml. of *3% hydrogen peroxide solution.* Shake vigorously and allow to settle until all effervescence ceases, since bubbles vitiate the fluorescence readings.

Fluorescence Reading. Pipet 15 ml. of the solution into the cell of the fluorophotometer and read the fluorescence, after adjusting the instrument, using a Corning glass filter No. 511 for transmitting the incident light and No. 351 for the fluorescent light, both filters being 2 mm. thick. Compare with a graph or directly with a standard solution.

Standard Solution. Treat a 15-ml. aliquot of the standard solution (1 ml. = 0.0856 γ) with 1 ml. of *4% permanganate solution* and 3 ml. of *3% hydrogen peroxide* (diluted

Superoxol). Pipet 15 ml. of this mixture (= 1 γ of riboflavin) into a cell and determine the fluorescence.

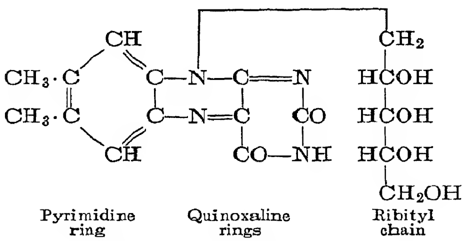
Blank. Correct the standard for the fluorescence obtained in a blank determination using the same amount of the reagent as in an actual analysis. Run also a complete blank for all reagents used in the determination of both vitamins.

Mackinney and Sugihara Modification. See Part II, C1, Riboflavin.

(Vitamin B_2 or G)

The role of riboflavin in nutrition is not sharply defined, but it is believed to act in conjunction with thiamin as a preventive of certain nervous disorders, although it is not the true anti-pellagra factor. Liver, fish roe, and yeast are potent sources of the vitamin; meat, milk, fish, eggs, wheat germ, and different types of vegetables are somewhat less valuable sources.

The structural formula shows that the molecule consists of alloxazine joined to a ribityl sugar chain thus:



RIBOFLAVIN (Karrer *et al.*)

The vitamin is obtained as orange-yellow crystalline needles, slightly soluble in water and insoluble in fat solvents.

Analytical Methods. See also Part II, A1, C1, G1, H1, and K1.

The chemical methods for the determination of riboflavin which follow depend primarily on (1) the fluorescence of riboflavin, (2) the color absorption of riboflavin, after destruction of foreign colors by oxidation, or after destruction of the riboflavin and subtraction of the absorption of the residual color from the total absorption, (3) the color absorption of lumiflavin formed from riboflavin by irradiation, (4) the influence of riboflavin on bacteriological growth, and (5) the influence of riboflavin on enzymic action.

The fluorescence of riboflavin is perhaps its most characteristic property as yet utilized in the determination of the vitamin. Although foreign colors may be more or less completely destroyed by preliminary oxidation, it is uncertain whether riboflavin and its relative, lumiflavin, are the only constituents of foods with similar fluorescence.

Cohen Mercury Lamp Fluorometric Method. Cohen, a pioneer in the study of the fluorescence of riboflavin, at first used the Kleinmann nephelometer.[111] Later[112] he employed his own apparatus. The light from a mercury lamp is passed through a nickel monoxide filter and allowed to fall on the solution; then it is passed through a selenium cell and an untra-violet filter and directed onto a Spiegel galvanometer with a sensitivity of 1 mm./(3×10^{-9} amperes) at a scale distance of 1 mm. After standardization with the fluorescein, the fluorescence of lactoflavin (riboflavin) is determined at a concentration of 0.97×10^{-5} g./ml. The fluorescence of fluorescein multiplied by 1.8 gives the fluorescence of riboflavin.

von Euler and Adler Acetone Extraction Quartz Lamp Fluorometric Method.[113] APPARATUS. *Pulfrich Photometer*, equipped for measuring the fluorescence with the analytic quartz lamp (Zeiss).

PROCESS. *Extraction.* Run the sample through a meat chopper or cut into small pieces with shears. Extract a weighed portion for 24 hours in the cold with 10 to 20 times its weight of *80% acetone,* decant the supernatant liquid, and repeat the extraction

twice, using 10 times the original weight of cold *60% acetone*. Remove fat from the combined extract with *naphtha* by shaking out small amounts directly and large amounts after evaporation *in vacuo*. Wash the naphtha extract with water and add to the main extract.

The united aqueous phase contains the total lyochromes (flavins) which are characterized by the fluorescence observed in the condensed light of an incandescent carbon light.

Fluorescence Reading. Compare in the photometer the fluorescence of the unknown at *p*H 7 with that of a series of solutions prepared by dilution of a standard solution and treatment in the same manner as the unknown.

CLASSIFICATION. von Euler and Adler classify animal tissues according to the riboflavin content into 6 groups, containing in the fresh material, as found by them, as follows: I (liver, kidney), 10 to 20; II (adrenals), 5 to 10; III (brain), 1 to 5; IV (lungs), 0.5 to 1; V, 0.025 to 0.5; and VI (blood), 0.025 γ/g.

Narasimhamurthy Adsorption Fluorometric Method.[114] APPARATUS. *Colorimeter* with *Quartz Cups* and *Quartz Mercury Vapor Lamp*.

PROCESS. *Extraction*. Extract in a 250-ml. volumetric flask the finely divided material, containing not over 200 γ of the vitamin, for 48 hours at 37° with 50 ml. of acidified *20% methanol* (0.1 *N* with hydrochloric acid), and centrifuge. Shake well, digest at 37° for 48 hours with occasional shaking, transfer to a centrifuge tube, centrifuge, and if necessary add 1 ml. of *hydrochloric acid* to precipitate protein, etc. Transfer the supernatant liquid to a 250-ml. flask, wash the residue with *strong methanol* until all color is removed, reduce the combined extract and washings to 40 ml. *in vacuo* at 50° with occasional bubbling of air to prevent reduction of the flavin.

Adsorption. To the clear solution, add 3 g. of *fuller's earth* and 3 ml. (2 ml. if 1 ml. was added earlier) of *hydrochloric acid* and, after shaking moderately for 1 hour, centrifuge.

Color Formation. Shake the adsorbate for 1 hour with 50 ml. of a *1 + 1 + 4 methanol pyridine-water mixture* in a 250-ml. flask, centrifuge, and wash the residue with *methanol* until all color is extracted. Distil the combined extract and washings *in vacuo* to a volume of about 2 ml., transfer to a centrifuge tube with *acetone* (not over 60% concentration), centrifuge, and wash the residue.

Color Reading. To the combined supernatant liquid and washings, add *alkali* to *p*H 7.0, centrifuge if insoluble matter separates, and compare the fluorescent color of the clear solution in a colorimeter with a *lactoflavin standard*, using quartz cups and a quartz mercury vapor lamp.

Light, except for an occasional dull red, should be excluded at all times.

Weisberg and Levin Adsorption Fluorometric Method.[115] The method is simple and requires no expensive apparatus.

APPARATUS. *Ultra-violet Lamps*. Nine Blue Glow argon mixed gas lamps (General Electric Vapor Lamp Company, Hoboken, N. J.) mounted in a box and wired as shown in Fig. 77. The cover of the box (Fig. 78) consists of a light filter 16.25 cm. square of Corning glass, Violet Ultra, No. 586 (Corning Glass Works, Corning, N. Y.) which transmits a nearly monochromatic beam at 3650 Å. Solutions of the fluorescein standard and the unknown are placed on the filter.

French Square Bottles, 60 ml.

REAGENTS. *Standard Sodium Fluorescein Solution*. Standardize against pure riboflavin.

Other reagents as in the Narasimhamurthy method.

PROCESS. *Extraction*. Follow one of two procedures:

(A) Reflux a weighed portion of the sample three times with *methanol acidulated with acetic acid* in the presence of *carbon dioxide* and the absence of light, washing the residue

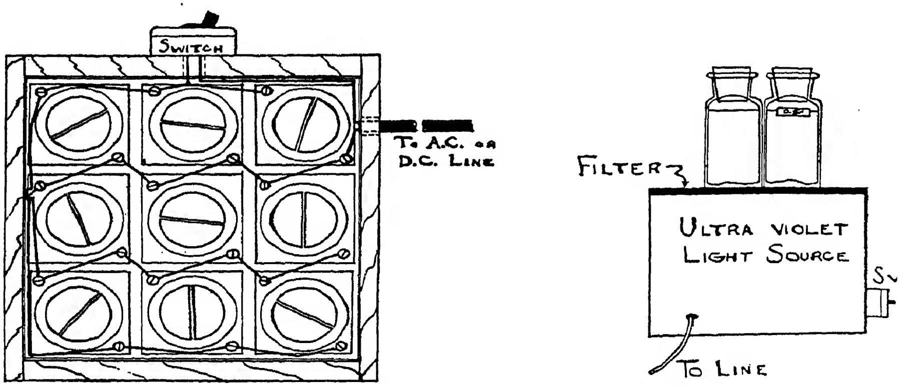
with fresh *methanol* between extractions. Add one volume of *acetone* and keep in the refrigerator at −17.78° overnight to flocculate impurities. Filter, evaporate *in vacuo*, adding water from time to time, and make the methanol-free liquid up to a definite volume. Filter and defat if necessary.

(B) Reflux as under (A). Adsorb the riboflavin from the methanol on *fuller's earth* (10 g. per 453.6 g.), assuming a potency of whey powder or dried yeast. Centrifuge, wash the earth, and elute with *1 + 1 + 4 pyridine-methanol-water mixture* (36 ml. per gram of adsorbate). Repeat the elution, filter, combine the eluates, condense *in vacuo*, and proceed as under (A).

Conduct a blank in like manner and introduce a correction for its fluorescence.

Reading. Compare with standards (50 ml.) in French square bottles containing 0.1 to 1.0 γ/ml. of riboflavin. Dilute the unknown so as to fall between the range of the standards.

Hodson and Norris Fluorometric Method.[116] In this method the fluorescence is measured in a fluorometer. Another departure from the von Euler and Adler method is the reduction of riboflavin to a non-fluorescing form by sodium hydrosulfite (not by stannous chloride), a feature shared by the Lunde, Kringstad, and Olsen method. Like the latter, it is an indirect procedure, as other pigments and fluorescing substances are present in foodstuffs. Although details were worked out at the Department of Poultry Husbandry of Cornell University, they are applicable to a wide variety of foods and the results agree closely with those by the Snell and Strong Bacterial method.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1937, 9, 524

FIGS. 77 and 78. Weisberg and Levin Ultra-violet Riboflavin Apparatus.

APPARATUS. *Fluorometer*, Pfaltz and Bauer; also accessories as follows: (1) *Corning Filter*, H.R. lantern blue, No. 554; (2) *Optical Cell*, 50 mm. cubical; (3) *G. E. Mercury Vapor Lamp*, Type H3, 85 watt, stabilized with Raytheon voltage regulator, Type V4, (4) *General Radio Variac Transformer*, Type 200 CMH, 115-volt circuit, to be placed between the voltage regulator and lamp transformer to step up the voltage, and (5) *Galvanometer*, Lange multiflex.

REAGENTS. *Standard Riboflavin Solution*. Dissolve a weighed amount of riboflavin of highest purity in distilled water and make up to a known volume. A concentration of 50 γ/ml. is suitable. Keep in a cool dark place. Add a few drops of glacial acetic acid

to insure an acid pH and aid in stabilization.

Sodium Hydrosulfite Solution. Dissolve 1 g. of sodium hydrosulfite ($Na_2S_2O_4$) and 1 g. of $NaHCO_2$ in 20 ml. of ice-cold distilled water. Keep in ice bath. Stable only about 4 hours.

Stannous Chloride Solution. Dissolve 10 g. of $SnCl_2 \cdot 2H_2O$ in 25 ml. of HCl and store in a brown, glass-stoppered bottle. For the determination, dilute each day as needed 1 ml. of this stock solution to 200 ml. with water.

STANDARDIZATION OF FLUOROMETER. It is essential to regulate the intensity of the activating light so as to obtain a standard galvanometer response, proceeding as follows. Insert a cube of canary glass (which fluoresces in the green portion of the spectrum) in the path of the activating light at such a position in front of the photoelectric cell that the galvanometer light beam comes to rest at an intermediate point on the scale. The position must be easily reproducible for future use. A solution of riboflavin or sodium fluorescein may be used in place of the canary glass cube.

Standard Curve. Obtain the galvanomic response to a series of riboflavin solutions of known concentration, prepared by diluting the standard solution with distilled water, from 0 to that which gives the full scale deflection of the galvanometer. Plot the concentrations of riboflavin in gammas per milliliter against the galvanometer response and use the curve for conversion of the galvanometer readings on the unknown solution.

PROCESS. *Extraction.* Weigh 5 g. of the sample into a 500-ml. Erlenmeyer flask, add 50 ml. of *0.25 N sulfuric acid*, and mix.

For alfalfa meals, etc., use double the quantity of *0.25 N sulfuric acid;* for milk by-products containing casein, extract with *1.0 N sulfuric acid-acetone solvent (1 + 3)* instead of the sulfuric acid.

Reflux under a condenser for 1 hour, allow to come to room temperature, then bring to pH 7 to 7.5 with *6.5% trisodium phosphate solution*, using *nitrazine paper* as indicator. Add water sufficient to make the total additions 50 ml., which makes the total volume of liquid added to the sample 100 ml., corresponding to a dilution of 1 + 20. Let stand 30 minutes, filter through pleated paper, and pipet an aliquot into a 200-ml. volumetric flask.

For molasses, add 90 ml. of *methanol* slowly to 10 ml. of the neutralized extract at pH 7 to 7.5 and filter, avoiding evaporation. Transfer a suitable aliquot to a 200-ml. volumetric flask. .

Reduction. Dilute the aliquot to approximately 175 ml. with water, add 2 ml. of *sodium hyposulfite solution* and 2 ml. of *stannous chloride solution*, mix, and make up to the mark. After allowing to stand 10 minutes, pour into a 1-liter Erlenmeyer flask, shake vigorously for 5 minutes with access to air.

Pipet a definite volume of the solution into the optical cell, the amount depending on the concentration of riboflavin and the amount of light-absorbing impurities.

Fluorescence Reading. Adjust the intensity of the mercury vapor light by the variable transformer so that the canary glass cube gives the standard response. Place the optical cell containing the solution of the unknown in the fluorometer and make an initial reading (a). Add a known amount of the standard riboflavin solution (not more than 2% of the volume of the unknown) and make a second reading (b). Reduce the riboflavin in the solution by adding *sodium hyposulfite solution* (2% of the volume of the unknown) and make a third reading (c). Add the same amount of *standard riboflavin solution* to a volume of water equal to that of the unknown solution and make a fourth reading (d).

CALCULATION. Convert the galvanometer readings into gammas of riboflavin per milliliter, by referring to the standardization curve, and calculate by the following formula:

$$R = (a' - c'y)\,\frac{d'}{b' - a'}\,z$$

in which R is the corrected gammas per gram of riboflavin a', b', c', and d', the converted first, second, third, and fourth readings respectively; y is the correction factor to compensate for the dilution resulting from the addition of standard riboflavin and sodium hyposulfite solutions; $a' - c'y$ is the uncorrected gammas per milliliter of riboflavin of the unknown solution; $d'/(b' - a')$ is converted readings d' divided by the converted reading b' minus the converted reading a', thus correcting for the absorbing effect of the stable interfering pigment upon the activating and fluorescent light; and z is the dilution factor.

EXAMPLES. The following results (among others) were obtained by the fluorometric, microbiological, and absorption photometric methods respectively: dehydrated alfalfa meal 15.5, 15, and . . .; white fish meal 10.6, 11, and 9.7; liver meal 78.8, 72.0, and 74.5; dried skim milk 20.0, 17, and 21.4; yeast 32.4, 36, and . . .; soy bean oil meal 4.6, 4, and 4.7; and whole wheat 0.8, . . ., and . . . γ/g.

Other Fluorometric Methods. See Part II, A1, G1, and H1.

Lunde, Kringstad, and Olsen Oxidation-Reduction Colorimetric Method.[117] So far as the determination of the color absorption after oxidation with potassium permanganate is concerned, the method (Canning Laboratory, Stavanger, Norway) is essentially the same as the van Eekelen and Emmerie method (see Part II, G1), but the alternate procedure of reading the color absorption before and after reduction with sodium hyposulfite and obtaining that due to the vitamin by difference is a new departure, particularly valuable when difficultly oxidizable foreign colors are present.

APPARATUS. *Pulfrich Photometer*, with filter VS 45 and 3-cm. cell.

PROCESS. *Solution.* To 20 to 50 g. of the sample, containing at least 50 to 60 γ of riboflavin, add 100 to 250 ml. of *ethanol* and heat for 10 to 15 minutes on the water bath. Decant the supernatant liquid on a filter and repeat the extraction twice, using *70% ethanol*. Evaporate the combined extracts at 50° *in vacuo* to 100 to 125 ml., add *hydrochloric acid* to pH 3, then remove fat and color by shaking out with *ether*.

First Oxidation. Add to the aqueous solution 6 ml. of *4% potassium permanganate solution*, allow to stand 10 minutes, then destroy the excess of permanganate with *6% hydrogen peroxide solution*.

Adsorption and Elution. Shake the solution with a suitable amount of *frankonite* for 1 hour, collect the adsorbate on a filter, wash with water, and elute the pigments by heating with 50 ml. of *1 + 2 + 3 pyridine-methanol-water solvent* for 1 hour on the water bath. Filter, wash the frankonite with 15 ml. of the *mixed solvent*, and note the exact volume. Remove two portions (A and B) of 20 ml. each. Reserve portion B for direct color reading before and after reduction.

Second Oxidation. To portion A, add 2 ml. of *acetic acid* and 2 ml. of *saturated potassium permanganate solution*, shake well, allow to stand 10 minutes or until the oxidation is complete, then add 1 ml. of *6% hydrogen peroxide solution*, to destroy the excess of permanganate, and filter.

Color Reading After Oxidation. Remove the filtrate to a 3-cm. cell of the Pulfrich photometer and read, using light filter VS 45.

Determinations by Lunde *et al.* on 2 pure preparations of riboflavin from different sources gave E values for 100 γ/ml. dilution in a 1-cm. cell of 3.03 and 3.08, aver. 3.055, equivalent to 9.165 in a 3-cm. cell. This value should be checked by the analyst and the amount of riboflavin present in the solution examined calculated by rule of three.

Color Reading After Reduction. Read in like manner portion B, then convert the riboflavin into a colorless compound by adding to the solution while in the colorimeter cell a small amount of *crystalline sodium hydrosul-*

fite ($Na_2S_2O_4$) until no further color change is evident, taking care that the solution is neutral or faintly alkaline. Subtract the final reading from the reading before reduction, thus obtaining the color due to riboflavin alone.

Examples. In 25 g. of spinach 87.5 γ were found by the oxidation method (A) and 89 γ by the reduction method (B). After adding 100 γ of riboflavin, the results were 186 and 184 γ respectively.

In the presence of pigments other than riboflavin not destroyed by oxidation, the second procedure is the more accurate, assuming that the foreign coloring matter is not reversibly reduced.

Other Colorimetric Methods. See Part II, G2, H1, and K1.

Kuhn, Wagner-Jauregg, and Kaltschmitt Irradiation Colorimetric Method.[118] The originators (Heidelberg) of this method took advantage of the discovery of Warburg and Christian[119] that *riboflavin* is converted by irradiation into a "photoderivative," now known as *lumiflavin,* which is separated from foreign colors by reason of its solubility in chloroform.

Apparatus. *600-Watt Lamp.*

Pulfrich Photometer (Zeiss), with color filter S 47 and 3-cm. cell.

Process. *Extraction.* Vary the extraction of the vitamin according to the product. The instructions are somewhat vague, but the following seem consistent.

Pulp tomatoes, carrots, spinach, and potatoes, add 3 parts of water and a little *hydrochloric acid,* boil for 2 hours, cool, and centrifuge; then extract the residue by long shaking at 35° with *80% methanol,* combine the methanolic and aqueous extracts, and concentrate *in vacuo.* Extract wheat bran for 12 hours at 20 to 30° with 4 parts of *80% methanol,* then concentrate *in vacuo.* Heat malt extract on a steam bath for 4 to 5 hours with 3 to 4 parts of water, acidify with *hydrochloric acid,* centrifuge, and decolorize the extract with *1.0 N potassium permanganate* at 15 to 20°.

Whichever the method of extraction, add *5 N sodium hydroxide solution* to 0.5 normality and remove the fat by shaking out with *chloroform.*

Irradiation. Cool the alkaline solution to 20° or less and irradiate for 2 hours with a 600-watt lamp at a distance of 20 to 30 cm. Acidify the irradiated solution with *acetic acid,* shake out the lumiflavin with at least 3 portions of *chloroform,* and dry the chloroform extract by means of *anhydrous sodium sulfate.*

Color Reading. Concentrate to a suitable volume and measure the color absorption of the chloroform solution in a Pulfrich photometer, using color filter S 47.

Calculation. The E value (absorption coefficient) for pure lumiflavin (100 γ/ml. of chloroform) is 4.30; the factor for conversion of lumiflavin into riboflavin is 1.47, which is 376 (the mol. wt. of riboflavin) divided by 256 (the mol. wt. of lumiflavin).

Examples. Among results reported by Kuhn *et al.* on riboflavin are the following: orange juice 0.069 to 0.089, tomato pulp 0.71, carrots 0.20, spinach 0.57, dry alfalfa 7.17, dry hay or grass 1.42, potatoes 0.08, wheat bran 0.33, milk 1.0, dry yeast 18.0, and beef liver 15.9 γ/g. or γ/ml. Their recoveries from 0.12 to 3.72 mg. of riboflavin ranged from 42 to 87%. As shown by van Eekelen and Emmerie[120] and admitted by Wagner-Jauregg[121] the irradiation, as practiced, was not complete.

Reindel and Fleischmann Modification.[122] The details (Institut of Technology, Weihenstephan) for yeast are doubtless applicable to other foods.

Apparatus. *Irradiator.* As designed by the authors, the apparatus consists essentially of two 100-watt incandescent bulbs, each with a reflector that concentrates the light on the extract of the sample placed between them. A stream of water passing through a

coil immersed in the liquid keeps the temperature below 20°.

Pulfrich Photometer (Zeiss), with light filter No. 47.

Reagents. *Standards.* Prepare 4 solutions containing from 1 to 1.5 mg. of pure riboflavin in 50 ml. of water. Irradiate in alkaline solution, acidify, shake out with chloroform, and determine the light transmission in 0.5- to 3-ml. cells as in the actual analysis and plot a curve with concentrations as abscissas and transmission (J) as ordinates.

Process. *Extraction.* Boil 200 g. of *yeast* (dry matter 25%) for 10 minutes with 200 ml. of water, cool, centrifuge, and decant off the liquid. Repeat the boiling and centrifuging twice, using smaller amounts of water, combine the liquids, add *0.5 N sodium hydroxide solution* to alkaline reaction, and make up to a convenient volume, then remove aliquots of 50 ml. or concentrate to 50 ml. *in vacuo.*

Irradiation. Place the solution in the irradiator, cool to below 20° and irradiate for 2 hours, thus converting the riboflavin into an equivalent amount of lumiflavin.

Chloroform Extraction. Add *acetic acid* to the irradiated solution to acid reaction and shake out with *chloroform.* Dry the chloroform extract with *anhydrous sodium sulfate* and concentrate by distillation to a volume of exactly 50 ml.

Light Transmission. Transfer a portion of the chloroform solution to a 3-cm. cell of the Pulfrich photometer and determine the light transmission in terms of J on the scale.

Calculation. For most purposes sufficiently accurate results are obtained by comparison with the standard curve, but for greater exactness calculate by the formulas as follows.

Obtain the value for E (transmission) for each reading of the standard solution (4 readings for each of the 4 concentrations) by the formula

$$E = \log \frac{100}{J}$$

Then obtain the K value (extinction constant) by the formula

$$K = \frac{E}{c \times d}$$

in which c is concentration in milligrams per 50 ml. and d is the cell thickness in centimeters. As obtained by Reindel and Fleischmann, the values for K ranged usually from 42 to 45.

The milligrams (c') of the vitamin in the solution of the unknown is obtained by the formula

$$c' = \frac{}{K \times d'}$$

in which J' is the reading and d' is the cell thickness in centimeters of the unknown.

Snell and Strong Bacteriological Method.[123] The results of investigations carried out at the Wisconsin Experiment Station [124] show that riboflavin is essential for the growth of certain lactic acid bacteria and the amount of growth in some cases is directly proportional to the concentration of the vitamin.[125] The method is rapid, requires only a small amount of the material, and gives higher results than several other methods; it requires, however, bacteriological equipment and knowledge of bacteriological technique.

Apparatus. *Autoclave, Incubator, Bacteriological Test Tubes* 16 to 20 x 150 mm., *100-watt bulb,* and minor pieces of apparatus.

Evelyn Photoelectric Colorimeter, with 540-mμ filter.

Galvanometer.

Reagents. *Stock Culture and Inoculation.* The organism is *Lactobacillus casei* which is probably identical with *Bacillus casei* ϵ of Freudenreich; it is carried in yeast-water agar containing 1% of dextrose. Make a series of stab transfers (b, c, d, etc.) from the original culture (a) into yeast water-dextrose agar.

Incubate 24 hours at 37° and store in a refrigerator. Reserve at least one tube (*b*) as the stock culture and use the other tubes (*c*, *d*, etc.) to prepare the inoculum as described below.

When determinations are made on several succeeding days, it is not necessary to grow inoculum from stock cultures (*c*, *d*) each day, since a drop of inoculum *e* (derived from *c*) can be transferred to a similar tube (*f*) which is incubated for use the next day. Do not use inoculum cultures after they are 36 hours old and always return to a stock culture about every fifth day to avoid contamination and bacterial variation. Prepare new stock cultures corresponding to *b*, *c*, and *d* at monthly intervals from a tube such as *b* of the preceding month.

Photolyzed Sodium Hydroxide-Treated Peptone Solution. Expose to light from a 100-watt bulb with reflector at a distance of about 30 cm. for 6 to 10 hours in a 25-cm. crystallizing dish a mixture of 40 g. of peptone (Difco Bacto) in 250 ml. of water and 20 g. of NaOH in 250 ml. of water, allow to stand at room temperature for an additional 14 to 18 hours (total of 24 hours), neutralize with glacial acetic acid (27.9 ml.), add 7 g. of anhydrous $NaC_2H_3O_2$, and dilute to 800 ml. Preserve under toluene.

Cystine Solution. Prepare a solution of cystine hydrochloride containing 1 mg. of cystine per milliliter. Keep under toluene.

Solutions of Inorganic Salts. *A*. Dissolve 25 g. of K_2HPO_4 and 25 g. of KH_2PO_4 in 250 ml. of water.

B. Dissolve 10 g. of $MgSO_4 \cdot 7H_2O$, 0.5 g. of NaCl, 0.5 g. of $FeSO_4 \cdot 7H_2O$, and 0.5 g. of $MnSO_4 \cdot 4H_2O$ in 250 ml. of water.

Yeast Supplement. To 100 g. of Bacto-yeast extract (Difco Laboratories, Detroit, Mich.) in 500 ml. of water, add 150 g. of basic lead acetate (Horne's sugar reagent) dissolved in 500 ml. of water, and filter. Add to the filtrate NH_4OH to about *p*H 10 and again filter. Acidify the filtrate with glacial acetic acid, precipitate the excess of lead with H_2S, filter, and discard the precipitate. Make up the filtrate to 1000 ml. and store under toluene in the refrigerator; 1 ml. = 100 mg. of the original yeast extract.

Basal Medium. Mix 50 ml. of the peptone solution, 50 ml. of cystine solution, 5 ml. of yeast supplement, 5 g. of dextrose, 2.5 ml. of solution *A*, and 2.5 ml. of solution *B*, adjust to *p*H 6.6 to 6.8 with NaOH, and dilute to 250 ml.

Inoculation Suspension. Stab with a stock culture a sterile tube of the basal medium containing 0.5 to 1.0 γ of riboflavin per 10 ml. Incubate 24 hours at 37°, centrifuge aseptically, and resuspend in an equal volume of sterile 0.9% sodium chloride solution.

Standard Riboflavin Solution. Prepare a stock solution by dissolving 10 mg. of pure crystalline riboflavin in 100 ml. of warm 0.02 *N* acetic acid. Keep under toluene in a refrigerator. Dilute each day 10 ml. of the stock solution to 100 ml. with 0.02 *N* acetic acid; 1 ml. contains 10 γ of the vitamin.

STANDARD CURVE. For each set of assays, construct a standard curve for titration or growth data from a set of duplicate tubes containing 0, 0.05, 0.075, 0.1, 0.15, and 0.2 and single tubes containing 0.3 and 0.5 γ of riboflavin per 10 ml. of medium.

PROCESS. *Extraction.* Autoclave finely divided natural products for 15 minutes with a large volume of water at 1 kilo per sq. cm. or boil with *0.1 N hydrochloric acid* and subsequently neutralize, in which case not over 50 mg. of *sodium chloride* should be added to the assay tubes of the aliquot to be analyzed.

When large amounts of light-absorbing impurities (molasses) are present, add slowly to 10 ml. of neutralized extract 90 ml. of *methanol* and filter, avoiding evaporation. Transfer a suitable aliquot to a 200-ml. volumetric flask, dilute to 175 ml., and follow the usual procedure.

Dilute milk with distilled water and use an aliquot.

Sterilization. Into each of 50 assay tubes, pipet 5 ml. of the *basal medium* and a suitable aliquot of the extract containing the riboflavin, and dilute where necessary so that the volume in each tube is 10 ml. Plug with cotton, sterilize in an autoclave for 15 minutes at 1 kilo per sq. cm., and cool.

Inoculation and Incubation. Inoculate each tube with 1 drop (about 0.05 ml.) of the *inoculation suspension* and incubate at 37 to 40° for 1 to 3 days.

Bacterial Response and Reading. Two methods are proposed: (I) measurement of the turbidity produced by growth of the organism and (II) (for highly colored or turbid extracts) measurement of the acid produced during growth.

I. Turbidimetric Procedure. Shake the assay tubes until all bacteria are uniformly suspended, transfer the medium and cells to the colorimeter tube, and read directly the percentage of transmitted incident light from the galvanometer adjusted to read 100 with uninoculated basal medium in the colorimeter tube. Keep off all overhead lights.

II. Acidimetric Procedure. Transfer the contents of the assay tubes to 125-ml. Erlenmeyer flasks with 10 to 20 ml. of distilled water and titrate to *p*H 6.8 to 7 with *0.1 N sodium hydroxide solution*, using *bromothymol blue* as indicator. Use a color-comparison flask to aid in recognition of the end-point which is reproducible to about 0.1 ml.

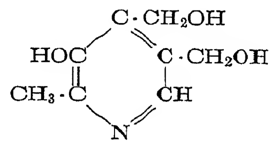
EXAMPLES. Results reported include milk *A* average 2, milk *B* average 1.69, and fresh beef liver average 42.1 γ/g.

Enzymic Method. See Part II, A1, Andrews, Boyd, and Terry Taka-Diastase Method.

ADERMIN OR PYRIDOXINE

(Vitamin B_6)

This anti-scorbutic factor, occurring like several other vitamins in yeast, liver, and cereal germs, was isolated by György in 1938, shown by Stiller, Keresztesy, and Stevens and by Harris, Stiller, and Folkers in 1939 to be 2-methyl-3-hydroxy-4, 5 (hydroxy-methyl)-pyridine, and synthesized by Harris and Folkers in the latter year.



ADERMIN (Stiller *et al.*)

The crystalline needles are readily soluble in water and ethanol, but only slightly in fat solvents. The vitamin is dispensed as the hydrochloride.

Analytical Methods. **Swaminathan Diazo-Sulfanilic Acid Colorimetric Method.**[126] As neither diazotized sulfanilic acid nor the phenol reagent is specific for vitamin B_6, Swaminathan devised the following method depending on the removal of interfering substances.

APPARATUS. *Colorimeter.*

REAGENT. *Diazotized Sulfanilic Acid Reagent.* Treat in an ice bath 2.5 ml. of a solution, containing 1.6 g. of $C_6H_7O_3NS \cdot H_2O$ and 45 ml. of HCl in 500 ml., with 0.4 ml. of 10% $NaNO_2$ solution, and make up to 10 ml. with water.

PROCESS. *Digestion.* Heat at 70 to 80° 2 to 50 g. of the finely divided sample, containing 50 to 100 γ of vitamin B_6, with 200 ml. of *0.1 N sulfuric acid* for 30 minutes, then cool and digest for 24 hours at 38° with 0.5 g. of *pepsin* dissolved in 10 ml. of water, and 1 ml. of *toluene*.

Removal of Proteins. Heat as before, cool, adjust to *p*H 7.2 by adding 100 ml. of *0.2 N barium hydroxide solution*, and remove the precipitate by centrifuging, washing with 100 ml. of water. Treat the solution with 50 ml. of *10% sodium tungstate solution* and 50 ml. of *0.67 N sulfuric acid* and again remove the

precipitate by centrifuging as before. Adjust the solution to pH 7.2 with *40% sodium hydroxide solution*, add 70 ml. of *1.0 N barium acetate solution*, and again centrifuge and wash with 50 ml. of water.

Removal of Purine, Pyrimidine, and Imidazole Bases. To the solution add 15 ml. of *1.0 N silver nitrate solution*, followed by 50 ml. of *0.2 N barium hydroxide solution*, then remove the excess of silver with *hydrochloric acid* and of barium with *sulfuric acid* respectively.

Adsorption. Dilute the clear centrifugate to 1 liter and shake for 5 minutes with 2 g. of *clarite.* Filter with suction, wash with 50 ml. of *0.01 N sulfuric acid* and 100 ml. of water, and dry in air at 95°. Heat 1.6 g. of the dry adsorbate at 70 to 80° for 15 minutes with 60 ml. of *0.1 N barium hydroxide solution*, cool, and remove the clay by centrifuging, washing with 20 ml. of water. Remove traces of purines, etc., as above, using 3 ml. of *0.1 N silver nitrate solution*, then remove the excess of silver and barium as before. Adjust to pH 6, evaporate to 20 ml., readjust to pH 7.2, filter, and make up to 40 ml.

Color Formation. Treat 10-ml. aliquots with 4 ml. of *50% sodium acetate solution* and 1 ml. of *diazotized sulfanilic acid reagent.* Add 2 ml. of *5.5% sodium carbonate solution.*

Color Reading. Determine the bright yellow color value by comparison against a *standard solution* of 20 γ of the pure vitamin treated in the same way.

Examples. Dried brewer's yeast 54, rice polish 13.4, sheep liver 13.4, sheep muscle 4.5, cow's milk 1.7, yellow maize 7.1, wheat 7.6, husked rice 6.6, soy bean 8.0, beet root 1.3, cabbage 3.1, and ripe plantain 1.3 γ/g.

Scudi Chloroimide Colorimetric Method.[127] The method, devised at the Merck Institute, employs the reaction of 2,6-dichloroquinone-chloroimide with the vitamin in the presence of a veronal buffer and a borate buffer that inhibits the formation of extraneous phenols. The practical application of the method to biological material is still under investigation.

Apparatus. *Bausch & Lomb Spectrophotometer* or *Evelyn Colorimeter.*

Reagents. *Chloroimide Reagent.* Dissolve 100 mg. of 2,6-dichloroquinone-chloroimide (Eastman No. 2483) in 1600 ml. of acid-free reagent grade normal butanol. Store in a brown bottle at a low temperature and warm to room temperature for use. The reagent keeps 2 weeks. With pure butanol, in the absence of the vitamin, using filter No. 660, taken as 100%, 14 to 16% absorption in control tests indicates satisfactory activity and 20% absorption is proof of deterioration. For half strength, dilute with an equal volume of butanol.

Veronal Buffer, pH 7.6. Dissolve 15 g. of sodium diethylbarbiturate (Merck) in 700 ml. of water and titrate to pH 7.6 with the glass electrode. Filter from the diethylbarbituric acid precipitate.

Borate Buffer. The usual Clark and Lubs buffer at pH 8.6.

Bromothymol Blue and Thymol Blue Solutions, outside indicators. Prepare each by dissolving 100 mg. of the dye in the minimum amount of ethanol, diluting to 1 liter with water, and adjusting to their mid-points with dilute acid or alkali.

Process. *Color Formation.* Adjust the aqueous solution of the vitamin to a pH of 6.8 to 7.2, using *bromothymol blue* as an outside indicator. Add to a neutral solution of the vitamin 1 volume of *veronal buffer* and 4 volumes of the *chloroimide reagent* and shake briefly but vigorously, repeating after 5 minutes, and after 15 minutes centrifuge.

Color Reading. Pipet 3 volumes of the supernatant liquid, containing the vitamin indophenol, into 1 volume of *absolute ethanol* and read the color value 50 ($\pm$ 5) minutes after addition of the reagent. Using the Bausch & Lomb spectrophotometer at 650 mμ, solutions containing 2 to 40 γ of the vitamin yield colors obeying Beer's law. If

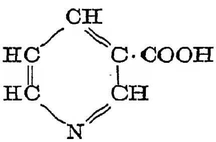
examined in the Evelyn colorimeter with filter No. 660, a solution containing 0.5 to 10 γ may be used.

Kühn and Löw Carbopyridinium–Cyanin Colorimetric Method.[128] Waisman and Elvehjem[129] discuss this method which does not appear to have been applied to biological material.

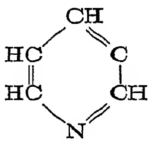
Nicotinic Acid or Niacin

The name niacin has been sanctioned by the U. S. Social Security Administration because of misconceptions arising from the use of nicotinic acid.

The vitamin has been classed by some in the B group and by others as vitamin PP. It has long been known as an organic chemical, but only recently has been established as the true anti-pellagra vitamin.



Nicotinic Acid



Nicoti

Yeast and liver serve for preparing concentrates. Whole cereals, liver, and white meat are especially valuable sources of the vitamin; milk, legumes, and leaf vegetables furnish good amounts.

It occurs both as the acid and the amide (nicotinamide).

The vitamin is obtained as white crystalline needles, melting at 235 to 237°, soluble in water and ethanol, but not in ether.

Analytical Methods. See also Part II, A2, G1, and H1.

Swaminathan Aniline–Cyanogen Bromide Colorimetric Method.[130] The reaction of the pyridine ring with cyanogen bromide and aniline, first applied by König[131] and later by Parry and Stafford-Jones[132] for the determination of pyridine in nicotine, has been adapted by Swaminathan to the determination of nicotinic acid in foods and biological products.

Apparatus. *Colorimeter* or *Nessler Tubes.*

Reagents. *Nicotinic Acid Solution.* Dissolve 10 mg. in water and make up to 1 liter; 1 ml. = 0.01 mg. = 10 γ.

Cyanogen Bromide Solution. Decolorize an ice-cold saturated aqueous solution of bromine by addition dropwise of ice-cold 10% NaCN.

Process. *Extraction.* Bring to a boil a weighed portion of the finely divided sample in water and, if rich in starch, heat further on a steam bath for 20 minutes. Centrifuge, decant off the liquid, and treat twice in the same manner. Remove protein from the combined extracts by precipitation at 60° with *solid normal lead acetate* $[Pb(C_2H_3O_2)_2 \cdot 3H_2O]$ and filtration. Cool, precipitate the excess of lead with *0.1 N sulfuric acid,* centrifuge, and wash. Adjust the *p*H to 6.0 and evaporate to small volume, then add *hydrochloric acid* to a concentration of 5% and boil for 30 minutes to decompose any nicotinamide present. Cool, adjust with *sodium hydroxide solution* to *p*H 10 and, if colored, boil with *charcoal.* Adjust to *p*H 7 and make up to the mark in a volumetric flask.

Color Formation. Pipet into 25-ml. volumetric flasks aliquots of the extract and of the *nicotinic acid standard solution* (= 0.02 to 0.05 mg.) and dilute to exactly 16 ml. Add to each 2 drops of *saturated aniline solution* and 4 ml. of *cyanogen bromide solution,* both freshly prepared. Allow to stand 2 minutes, then add 4 ml. of *aniline solution,* shake, and dilute to 25 ml.

Color Comparison. Remove a portion of the yellowish green liquid to the cell of a colorimeter or to a Nessler tube and compare the color with that of a standard solution treated in like manner.

In his preliminary paper, Swaminathan directed to extract the color with three successive 5-ml. portions of purified *amyl*

alcohol and determine the color value of the combined extracts.

Examples. See Waisman and Elvehjem Modification below.

Notes. Roos [133] found the Swaminathan method reliable.

Swaminathan [134] found that hot water, hot *aqueous or ethanolic sodium hydroxide*, and *hot aqueous or ethanolic sulfuric acid* serve equally well as solvents regardless of whether aniline or β-naphthylamine, following cyanogen bromide, is used to develop the color. The color in ethanolic solution, however, is two or three times more intense than in aqueous solution.

I. Kringstad and Naess Modification.[135] The details of the modification represent work carried out at the Research Laboratory of the Norwegian Canning Industry.

Apparatus. *Pulfrich Photometer* (Zeiss).

Reagent. *Phosphate Buffer.* Dissolve 17.6 g. of KH_2PO_4 and 6.67 g. of Na_2HPO_4 in water and make up to 250 ml.

Process. *Extraction.* Digest a weighed portion of the sample with stirring for 1 hour on a boiling water bath with 2 to 3 times its volume of water. Decant the supernatant liquid and treat the residue twice in like manner. To the united extracts, add *sulfuric acid* to 0.1 N and digest for 2 hours on a boiling water bath to split off the nicotinic acid amide combined as cozymase. Neutralize the solution with *barium hydroxide solution,* filter, and make up the filtrate to a definite volume containing at least 2 γ of nicotinic acid amide per milliliter.

(*a*) *Direct Solution.* To a 20-ml. portion of the solution in a 25-ml. flask, add from a pipet as a stabilizer 5 ml. of *phosphate buffer* at pH 6.1 and filter on a dry paper if a precipitate forms.

(*b*) *Hydrolyzed Solution* (Total Nicotinic Acid). To a second portion of 20 ml., add 15 g. of *barium hydroxide* per 100 ml. and heat for 2 hours on a boiling water bath, thereby converting the amide quantitatively into the free acid. Neutralize carefully with *sulfuric acid,* centrifuge, wash the separated barium sulfate with 3 portions of 25 ml. each of hot water, and bring the combined solution and washings to a fixed volume.

Color Formation. To 10 ml. of each solution (*a* and *b*), add 4 drops of *saturated aniline solution* and mix. Then add 0.35 ml. of *saturated cyanogen bromide solution* and mix again. The color of the nicotinic acid develops in 8 minutes and of the nicotinic acid amide in 23 minutes, both following Beer's law.

Color Reading. Read both solutions in a Pulfrich photometer, using No. 45 filter, against the extract without adding cyanogen bromide. Compare with curves obtained for pure nicotinic acid and its amide, showing 0 to 16 γ/ml. as abscissas (horizontal) and 0 to 1.40 ε as ordinates. The results on solutions *a* and *b* differ little from each other.

Examples. Kringstad and Thoresen [136] applied the method to various vegetable and animal foods with typical results as follows: maize kernel 1.3, potatoes 1.0, dried brewer's yeast 35.5, beef 4.9, beef liver 17.7, fowl 10 to 13, cod 1.7, cod liver 1.6, canned salmon 6, herring (male) 3.2 to 3.5, (female) 4.01, herring roe 2.36, herring milt 2.23, and milk 0.054 γ/g. or γ/ml. of nicotinic acid (free and combined).

II. Melnick and Field Modification.[137] In this modification are incorporated details developed at the Medical School of the University of Michigan.

Reagent. *Buffer Solution.* Mix 10 ml. of 85% H_3PO_4, 30 ml. of 15% NaOH solution, and 1960 ml. of water, then add 333 ml. of absolute ethanol. The solution has a pH of 7 and 7 ml. titrated with phenolphthalein as indicator are equivalent to 10 ml. of 0.05 N NaOH solution. These values correspond to those of the 6 ml. of cyanogen bromide solution plus 1 ml. of aniline solution 5 to 10 minutes after mixing at room temperature.

PROCESS. *Hydrolysis.* To the material containing 10 to 400 γ of nicotinic acid in a test tube with 10- and 15-ml. marks, add 5 ml. of *hydrochloric acid*, then water to the 15-ml. mark, and heat for 30 to 40 minutes in a boiling water bath with occasional stirring.

Charcoal Decolorization. Cool, restore the volume to 15 ml., dilute with 10 ml. of *absolute ethanol*, transfer to a 150-ml. Erlenmeyer flask, shake with exactly 200 mg. of *charcoal*, and filter. Neutralize an aliquot of 8.33 ml., using 1 drop of *phenolphthalein*, to pH 7. Bring to 10 ml. and remove 3-ml. aliquots for color formation.

Color Formation. To the first aliquot, add 7 ml. of the *alcoholic buffer solution*, then estimate the photometric density of the color remaining after the charcoal decolorization, treating as directed for the foregoing modification.

Use a second aliquot of the test solution for the chemical reaction and the maximum total photometric density measurement. Obtain by difference that resulting from the chemical reaction alone.

CALCULATION. Convert this last value into milligrams of nicotinic acid by the use of the K value obtained when 10 γ of the acid is added to a third 3-ml. aliquot of the test solution.

NOTE. In a later paper [138] they state that aniline should not be added to the blank test, since in the presence of cyanogen bromide it does not take part in interfering side reactions with substances in the hydrolyzates.

III. Waisman and Elvehjem Modification.[139] The procedure, as carried out at the University of Wisconsin, is simpler than that of Melnick and Field.

APPARATUS. *Evelyn Photoelectric Colorimeter.*

REAGENTS. *Cyanogen Bromide Solution, 4%.* Decolorize a cold saturated solution of bromine in the cold with 10% NaCN solution delivered from a buret, avoiding an excess. The solution is stable for a long time if kept in the refrigerator.

Ethanolic Aniline Solution, 4%. Dissolve 4 g. of freshly distilled aniline in absolute ethanol.

Buffer Solution. Dilute with 980 ml. of water a mixture of 15 ml. of 15% NaOH solution, 5 ml. of 85% H_3PO_4, and 167 ml. of absolute ethanol.

Darco. A charcoal supplied by the Coleman and Bell Co., Norwood, Ohio.

Standard Nicotinic Acid Solution. Prepare solutions in absolute ethanol and in water containing 50 γ of nicotinic acid per milliliter.

PROCESS. *Hydrolysis.* Weigh a charge containing 25 to 200 γ of nicotinic acid into each of two 15-ml. graduated centrifuge tubes. To one add 50 γ of *nicotinic acid* in water solution to act as recovery. Add to both tubes 5 ml. of *hydrochloric acid* and 5 ml. of water, then heat in a boiling water bath for 30 to 40 minutes with stirring.

Charcoal Treatment. Cool, make up to 15 ml., and transfer to 125-ml. Erlenmeyer flasks, rinsing with exactly 10 ml. of *absolute ethanol*. Add 200 mg. of *Darco*, shake, and filter through quantitative paper into dry 50-ml. Erlenmeyer flasks.

Neutralization. Transfer an aliquot of 8.33 ml. into a graduated centrifuge tube, add 1 drop of *phenolphthalein solution*, then *saturated sodium hydroxide solution* dropwise to pH 8.5. Using *bromothymol blue* as an outside indicator, adjust to pH 7.0 by adding *dilute hydrochloric acid*, avoiding a total volume of more than 10 ml.

Color Formation. To tubes 1 and 2 of eight colorimeter tubes add 1 ml. of *absolute ethanol* and 2 ml. of water, to tubes 3, 4, and 5 add 3-ml. portions of the unknown, to tubes 6, 7, and 8 add 3-ml. portions of the unknown and recovery. Add 7 ml. of *buffer solution* to tubes 1, 3, and 6. Of these tubes, 3 and 6 furnish independent blanks for residual color of the sample after decolorization with the

charcoal. Tube 2 serves as a blank on the reagents. To tubes 5 and 8 add 5 γ of *nicotinic acid* for determination of photometric density of this known amount of nicotinic acid. To each of the tubes, but 1 and 6, add 6 ml. of *4% cyanogen bromide solution*, followed rapidly by 1 ml. of *4% ethanolic aniline solution.*

Color Reading. Shake all the tubes and read in the colorimeter within 5 to 6 minutes after the addition of the aniline to the last tube, using filter 400 or 420 mμ. Read tubes 3 and 6 against tube 1 set at 100 and tubes 4, 5, 7, and 8 against tube 2, also set at 100, thus obtaining a double check.

CALCULATION. Employ the following formulas:

$$K_1 = \frac{5\ \gamma \text{ nicotinic acid}}{\text{log tube } 5 - \text{log tube } 4}$$

$$K_1 \times (\text{log tube } 4 - \text{log tube } 3)$$

$$K_2 = \frac{5\ \gamma \text{ nicotinic acid}}{\text{log tube } 8 - \text{log tube } 7}$$

$$X_2 = K_2 \times (\text{log tube } 7 - \text{log tube } 6)$$

in which X_1 is the nicotinic acid content of the aliquot of the sample.

$X_2 - X_1$ should equal 5 γ, since the 3-ml. aliquot of the neutralized filtrate represents one-tenth of the original sample, and the original recovery sample had 50 γ of nicotinic acid added. See Melnick and Field Modification above.

EXAMPLES. Beef liver 202, pork liver 282, beef muscle 48, pork muscle 53, smoked ham 83, cow's milk 8.2, dried egg white 52, polished rice 105(?), and yellow corn (acid hydrolysis) 107(?) γ/g. or γ/ml., fresh basis.

Bandier Metol-Cyanogen Bromide Colorimetric Method.[140] Bandier (Copenhagen) states that small amounts of pyridine picolinic acid, α-picoline, trigonelline, or methylpyridinium chloride do not produce color in the presence of monopotassium phosphate. Although quinoline, 2-methylquinoline, piperidine, pyrrole, pyridine, and furfuraldehyde interfere, Ashford and Clark [141] regard the Bandier method more accurate than the Swaminathan bromide-aniline or the Karrer and Keller dinitrochlorobenzene methods.

APPARATUS. *Pulfrich Photometer.*

REAGENTS. *Cyanogen Bromide Solution,* 4%. See Waisman and Elvehjem Modification above.

Metol Solution, saturated (about 5%). Dissolve the metol (p-methylaminophenol sulfate) in water by thoroughly shaking; avoid heat and light. It spoils in 2 to 3 hours.

PROCESS. *Extraction.* To 5 ml. of *4 N sodium hydroxide solution* in a 10-ml. glass-stoppered Jena volumetric flask, add 2.5 g. of the air-dry ground sample, close with cotton, and heat 30 minutes on a steam bath, shaking once or twice. Cool short of gelatinization, add 1 ml. of *hydrochloric acid,* stopper tightly, and turn the flask upsidedown twice. After cooling 1 minute, add 0.8 ml. of the *acid* and shake as before, then cool to 20° and add water to the mark. Shake well and centrifuge 10 minutes. Transfer to another tube 1 ml. of the clear brown liquid (= 250 mg. of the dry sample), add 9 ml. of *acetone,* close with a rubber stopper, shake vigorously, and centrifuge 3 to 4 minutes.

Pipet into a round-bottom flask 4 ml. of the acetone layer (= 100 mg. of the sample), add about 3 ml. of water, and evaporate the acetone, using suction but no heat other than the warmth of the hand.

Color Formation. Transfer with the aid of 5 ml. of *2% monopotassium phosphate solution* to a 20-ml. graduated flask, heat 5 minutes on a water bath at 75 to 80°, add 1 ml. of freshly prepared *4% cyanogen bromide solution,* allow to stand 5 minutes in a water bath, and cool under the tap to room temperature. Add 10 ml. of freshly prepared *5% metol solution,* make up to 20 ml. with water, and let stand 1 hour at room temperature away from the light.

Color Reading. Read the color value of the solution in a Pulfrich photometer (filter

S 43); also read the color value of a blank carried along with actual determination and containing the same amounts of *cyanogen bromide, monopotassium phosphate,* and *metol,* diluted to a volume of 20 ml.

Since the acetone extract transferred to water has a slight color, treat a second 4-ml. portion of the acetone layer used for the analysis as above, but make up to 20 ml. with water instead of metol. Measure the color with distilled water in the other cell (filter S 43) and subtract the value found from the first reading. From this result and the coefficient of extinction determined on a standard solution (0.1 mg. of nicotinic acid), calculate the weight of nicotinic acid in the sample.

Process for Small Amount. Place 0.5 ml. of *4 N sodium hydroxide solution* and 250 mg. of the dried sample in a graduated centrifuge tube. Close with cotton, heat 30 seconds on a steam bath, shaking once or twice, then add 0.18 ml. of *hydrochloric acid,* shake again, and cool. Dilute with water to 1 ml., add 9 ml. of *acetone,* and proceed as above.

Harris and Raymond Aminoacetophenone-Cyanogen Bromide Colorimetric Method.[142] This method, devised for urine analysis, has been studied and elaborated by Kodicek [143] at the University of Cambridge. Of several score of common substances present in animal and vegetable foods, only a few members of the pyridine group gave positive reactions with the reagent. The method is claimed to be three to five times as sensitive as the aniline and metol methods.

Apparatus. *Pulfrich Photometer* (Zeiss), with 3-cm. cell and blue filter No. S 47.

Reagents. *Standard Stock Nicotinic Acid Solution.* Dissolve 10 mg. in 10 ml. of water. Prepare weekly a 1 mg. per 10 ml. dilution.

Cyanogen Bromide Solution (Kulikov and Krestowosdwigenskaja).[144] To an ice-cold saturated aqueous solution of bromine, add dropwise an ice-cold 10% aqueous solution of KCN until just decolorized. Prepare daily.

p-Aminoacetophenone Solution, 5%. To 5 g. of *p*-aminoacetophenone, add 30 ml. of 32% HCl and dilute to 100 ml. with water.

Process. *Extraction.* Suspend in a small conical flask 0.5 to 2.0 g. of the minced meat or organs or the ground cereal product in a mixture of 5 ml. of water and 1 ml. of *40% sodium hydroxide solution.* Heat on a steam bath for 1 hour, avoiding excessive evaporation, thus converting pyridine nucleotides and possibly other derivatives of nicotinic acid into the free acid.

Purification. After cooling, add 40 ml. of *ethanol* and centrifuge, thus precipitating foreign substances, leaving the nicotinic acid in solution.

Adjustment of pH. Add cautiously *hydrochloric acid* to pH 6.5, using *bromothymol blue* as external indicator and 1 to 2 ml. of *5% sodium bicarbonate solution* as a buffer. Dilute with *ethanol* to 50 ml.

Color Formation. Prepare four graduated 15-ml. stoppered flasks (A, B, C, and X). To B and C add 0.2 and 0.4 ml. of *standard nicotinic acid solution* (= 0.02 and 0.04 mg. nicotinic acid). To all 4 flasks add 10 ml. of the extract of the unknown and heat on a water bath at 70 to 80° about 10 minutes. To A, B, and C add 2 ml. of *cyanogen bromide solution* and to X 2 ml. of distilled water, mix, and allow to stand 5 minutes on a water bath. Cool for 5 minutes in cold water in a dark room, then add to all four flasks exactly 0.4 ml. of *5% p-aminoacetophenone solution,* fill to the 15-ml. mark with *ethanol,* mix, stand in a dark room 5 minutes, and place at once in the 3-cm. cells of the photometer.

Color Reading. Read immediately with blue filter No. S 47, correcting for the blank (X).

Calculation. Plot a curve with amounts of nicotinic acid as abscissas and color values as ordinates or use the following formula:

$$\frac{6V}{B + C - 2A}$$

in which A, B, and C are the extinction values for the unknown (sample A) and for the unknown plus 0.02 mg. (B) and 0.04 mg. (C) of nicotinic acid, V is the final total volume after neutralization, and n is the number of grams of tissue used.

Illustration. Found: $A = 0.21$, $B = 0.63$, $C = 1.05$, $V = 50$ (neutralized extract made up to 50 ml.), and $n = 1$ (1 g. of fresh weight pancreas used).

$$\text{Nicotinic acid} = \frac{0.21}{0.63 + 1.05 - 0.42} \times 6 \times 50 \quad 50\ \gamma/\text{g. fresh material}$$

Examples. Ox liver 170, ox muscle 43, salmon 84, cod 30, herring roe 21, egg white < 0.5, egg yolk 10, milk 3, maize meal 10 to 41, potatoes 20, and spinach 17 γ/g. or γ/ml., fresh material.

I. Arnold, Schreffler, and Lipsius Modification.[145] This modification differs from the procedures of Harris and Raymond and of Kodicek chiefly in that (1) the vitamin is extracted by autoclaving, (2) *potassium phosphate-sodium hydroxide* buffer at pH 6.0 is used, except for low vitamin amounts, to insure comparable pH conditions, and (3) the color value of the cyanogen bromide-p-aminoacetophenone reaction mixtures is not determined directly in the Pulfrich instrument, but the color is extracted with *ethyl acetate* and the solution in that solvent is examined in the Pfaltz and Bauer fluorometer.

II. Jones Fluorometric Modification.[146] The modification consists essentially of (1) the preparation of a solution by hydrolyzing the sample with *20% sodium hydroxide solution*, addition of *60% sulfuric acid* to pH 4 to 5, precipitation of proteins, salts, and coloring matter with *acetone*, filtration, and evaporation, and (2) determination of the nicotinic acid by the Harris and Raymond method, except that color reading is made fluorometrically. Interfering colors may be further reduced by treating the acetone solution before evaporation with 0.2 g. of *Darco G-60* per 100 ml.

Daroga Phosphomolybdic Acid-Stannous Chloride Colorimetric Method.[147] The method was devised at the Imperial College of Science and Technology, London.

Apparatus. *Tintometer*.

Reagents. *Phosphomolybdic Acid Reagent.* Dissolve by warming 2.5 g. of $20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$ in 40 ml. of 10% HCl, cool, filter, and dilute to 50 ml. Store in dark not longer than 4 weeks.

Stannous Chloride Reagent. Dissolve 10 g. of $SnCl_2 \cdot 2H_2O$ in 25 ml. of HCl.

Process. *Phosphomolybdic Acid Precipitation.* Add 1 ml. of a solution, containing 0.1 to 1 mg. of nicotinic acid, to 0.2 to 0.3 ml. of *phosphomolybdic acid reagent* in a 30-ml. beaker. Add 0.1 ml. more of the reagent if the precipitate is large. Without filtering, dissolve the precipitate by heating and allow it to reprecipitate on standing 2 hours or longer at room temperature. Filter through a sintered glass filtering crucible (G4) and wash three times with 1-ml. portions of *5% acetic acid solution*. Suck dry for 2 minutes and dissolve the precipitate in 1 to 2 ml. of *0.1 N sodium hydroxide solution*, filter, and wash with 15 ml. of water at 25°, collecting the filtrate in the original beaker.

Color Formation. Add to the solution 1 ml. of freshly prepared *stannous chloride reagent* and make up to 50 ml. in a volumetric flask.

Color Comparison. After 5 minutes at 25° match in the tintometer, using for the comparison *standard solutions of pure nicotinic acid*.

Snell and Wright Bacteriological Method.[148] The details of manipulation presuppose knowledge of bacteriological technique.

Examples. By the method the following results were obtained: yellow corn 26.0, corn meal 6.0, wheat 67.0, wheat flour 52.0, rolled oats 10.0, milk 0.84, veal 500.0, pork liver 894.0, and boiled ham 200 γ/g. or γ/ml.

Nicotinamide

See also Part II, H1.

Karrer and Keller Dinitrochlorobenzene Colorimetric Method.[149] As was observed by Vongerichte,[150] substances containing the pyridine group yield with 2,4-dinitrochlorobenzene pyridinium salts which are decomposed by alkalies with formation of yellow-red derivatives of glutaconaldehyde. Fortunately animal products are usually free from other pyridine derivatives that would interfere with the determination of nicotinic acid; vegetable foods, however, may contain pyridine alkaloids that seriously vitiate the results.

Apparatus. *Leifo Step-Photometer* or an equivalent instrument.

Process. *Extraction.* Boil 300 to 500 g. of the finely divided material three times for 30 minutes each with 3 volumes of water and shake the combined extracts 2 or 3 minutes with *ether* to remove fat. Add to the solution *sulfuric acid* until 0.1 *N*, then boil gently for 30 minutes and neutralize (to *litmus*) with hot *barium hydroxide solution*. Filter, wash three times with a little hot water, evaporate the filtrate and washings to 50 to 100 ml., and extract *in vacuo* for 5 to 6 hours with *butanol*. Distil the butanol and extract the residue twice with small amounts of boiling water. Evaporate the aqueous extract *in vacuo* to dryness, take up in a mixture of *absolute methanol* and dry *benzene* (benzol), filter, and again evaporate the filtrate to dryness *in vacuo*.

Color Formation. Prepare a mixture of the completely dry residue with 3 parts of *2,4-dinitrochlorobenzene* by dissolving the dinitro compound in *methanol*, adding to the residue, and distilling the methanol. Melt the dry mixture by heating on the steam bath for 30 minutes, take up in *80% ethanol*, add a few drops of *10% alcoholic potassium hydroxide solution*, and make up to the mark in a volumetric flask.

Color Reading. Measure the color value of the liquid in a Leifo step-photometer or an equivalent instrument and determine the corrected amount of nicotinamide by reference to a plotted curve.

Examples. Horse muscle 46.6, cattle muscle 38.3, horse liver 160.0, cattle liver 93.0, cattle kidney 194.0, and baker's yeast 120 γ/g.

Note. Vilter, Spies, and Mathews,[151] in applying the method to 15 ml. of urine, decolorize with *Darco charcoal* (Coleman and Bell), filter, evaporate to dryness, and add 1 ml. of *1% methanolic 2,4-dinitrochlorobenzene* directly to 3-ml. aliquots without extraction with butanol. After allowing to stand 1 to 3 hours and evaporating to dryness, heating is limited to 10 minutes at 105°.

Pantothenic Acid

R. J. Williams, of the Oregon State College and later of the University of Texas, and associates, collaborating with Stiller and associates of the Research Laboratory of Merck & Co., Rahway, N. J., have placed this vitamin on a firm chemical foundation. It is α,γ-dihydroxy-β,β-dimethylbutyryl-β'-alanide as represented by the formula: $HOCH_2 \cdot C(CH_3)_2 \cdot CHOH \cdot CO—NH \cdot CH_2 \cdot CH_2 \cdot COOH$. Results by Lunde, Kringstad, and Jansen[152] indicate that the anti-gray hair factor is identical with pantothenic acid.

The methods of analysis await careful study.

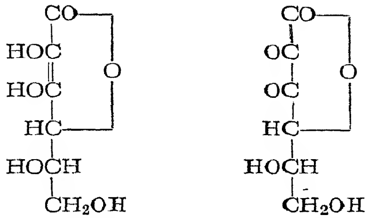
Ascorbic Acid

(Vitamin C)

It has long been known that citrus fruits, also sprouted cereals and legumes, are preventives of scurvy with its attendant structural changes in the bones, teeth, and blood vessels, which cause decay and hemorrhage.

In addition to citrus fruits, pineapples, strawberries, bramble berries, cucurbitaceous melons, and many leaf vegetables are excellent sources of the anti-scorbutic vitamin.

The formula below (Haworth, Hirst, *et al.*) shows how closely ascorbic acid is related to the hexoses.



l-ASCORBIC ACID — DEHYDROASCORBIC ACID

l-Ascorbic acid is a white powder, soluble in water, less so in ethanol, but insoluble in fat solvents, melting at 190 to 192°, and polarizing $[\alpha]_D^{20}$ + 21 to 22°.

d-Ascorbic acid has little or no antiscorbutic action.

Dehydroascorbic acid is formed by eliminating the hydrogens of the two hydroxyls in the ring of *l*-ascorbic acid and replacing the double bond by a single bond.

Analytical Methods. See also Part II, C1, D2, and G1.

Tillmans Indophenol Volumetric Method.[153] Zilva observed that phenol-indophenol is rapidly reduced in air to its colorless base by decitrated lemon juice with destruction of the antiscorbutic power of the juice. Tillmans *et al.*[154] (Frankfurt am Main University) soon after found that 2,6-dichlorophenol-indophenol (2,6-dichlorobenzenone-indophenol) has a similar action. By titrating with a standard solution of the dye, they obtained values closely paralleling those on vitamin C by animal tests.

The numerous modifications differ as to (1) the kind and strength of the acid solvent, (2) the reagent for removing proteins, cysteine, glutathione, glucic acid, tannins, and pigments, and (3) the treatment for reduction of dehydroascorbic acid or the prevention of its formation.

REAGENTS. *Solvents.* Tillmans used indiscriminately *acetone, 2 to 5% acetic acid, 2 to 3%* H_2SO_4, and even water alone. Greater discretion was exercised by (1) Emmerie *et al.*,[155] who chose *trichloroacetic acid*, (2) by workers who returned to *acetic acid*[156] or H_2SO_4,[157] or both, and (3) by Fujita and Iwatake and others[158] who adopted *2% metaphosphoric acid*, either alone or in conjunction with H_2SO_4 both as a solvent and an inhibitor of the dehydrogenation action of ascorbinase. Bessey and King[159] employ *trichloroacetic acid* for animal and *acetic acid* for plant extraction. Others combine *acetic acid* and *5%* H_2SO_4.

Standard Tillmans Reagent. The original reagent (0.01 *N*) was prepared from 2,6-dichloroquinone chloroimide. Later Tillmans dissolved commercial 2,6-dichlorophenol-indophenol in a phosphate buffer solution (1 part of monopotassium phosphate to 2 parts of dipotassium phosphate) at *p*H 7 and diluted the solution to 0.001 *N*. Although the directions for the method usually specify 2,6-dichlorophenol-indophenol, both Merck and Eastman list only the sodium salt. The molecular weight of the latter is about one-twelfth greater than that of the former; this is significant in the preparation of the standard solution. See Dewjatnin and Doroshenko Modification below.

PROCESS. *Solution.* Macerate 10 g. or other suitable quantity of the finely ground or pulped material in a mortar, best with the addition of *acid-washed sand*, with successive portions of the *acid solvent*. Make up to volume and remove an aliquot with a pipet. Details of technique are given under the modifications.

Defecation. Direct titration is satisfactory only for citrus juices and other materials free from interfering natural colors that re-

duce the dye. Emmerie and associates [160] clear with *mercuric acetate*, Dewjatnin and Doroshenko [161] with *lead acetate*, the excess of both reagents being removed by precipitation with *hydrogen sulfide*.

Reduction. Doubtless many of the early results are at fault because of the formation of the dehydro-acid during extraction. The original content may be restored by reduction with *hydrogen sulfide* or better retained by avoiding dehydrogenation by the use of *metaphosphoric acid* as the solvent.

Titration. Add *standard Tillmans reagent* to the aliquot from a buret without delay and carry the titration to the point where, as recommended by Bessey and King,[162] the blue color ceases to fade rapidly.

NOTES. Mottern, Nelson, and Walker [163] studied the action of the Tillmans reagent on reducing substances, such as glutathione, cysteine, and phenolic compounds, present in animal tissues; these are usually present in inconsiderable amount in vegetable products.

Bessey and King [162] mention cysteine, pyrogallol, glucic acid, and sugars (sucrose and glucose) heated with alkali as among the substances that cause slow fading to such an extent as to cause an error of 6 to 8% if the precaution is not taken to titrate to the end-point where rapid fading ceases.

Tillmans [164] removed interfering natural colors by shaking with *nitrobenzol*, in which the blue color is insoluble.

Kirk and Tressler [165] obtained a sharp end-point in the titration of the extract of fruits and vegetables, in the presence of colors, by the use of the Fischer electrometric titrimeter.

Dick [166] standardizes the *indophenol reagent* as follows: Mix carefully 10 ml. of the dye solution, 3 ml. of fresh 10% potassium iodide solution, and 2 ml. of 32% sulfuric acid. When the blue color has changed through red to yellow, add 60 ml. of water and titrate with 0.01 N thiosulfate solution; 1 ml. = 0.88 mg. of ascorbic acid.

I. Emmerie and van Eekelen Volumetric Modification.[167] This modification is one of several contributions from Utrecht University.

PROCESS. *Solution*. Mix 10 g. of the finely pulped fruit, vegetable, or animal tissue with acid-treated finely ground *sand*. Grind the mass in a mortar with successive amounts of a mixture of 1 part of *10% trichloroacetic acid* and 2 parts of water, centrifuging after each addition and decanting into a 50- or 100-ml. volumetric flask until the liquid nearly reaches the mark.

To the combined extracts, add 0.5 g. (more if necessary) of *calcium carbonate* and shake until the solution colors *Congo red paper* a faint violet. Make up to the mark, shake, and filter through a dry paper.

Defecation. To an aliquot of 10 ml. of the extract, add from a pipet or buret with stirring 5 ml. of *20% mercuric acetate solution*. If precipitation is not complete, run in an additional amount accurately measured. Centrifuge, carefully and completely pour off the supernatant liquid, and deliver into it a stream of *hydrogen sulfide gas* for 10 to 30 minutes. Filter to remove the mercuric sulfide, allow to stand overnight, drive out the excess of hydrogen sulfide with a stream of *nitrogen* (or *carbon dioxide*), testing with *lead acetate paper*, make up to 25 ml. in a volumetric flask and filter through a dry paper.

The hydrogen sulfide acting best at over pH 6.0, according to Fujita and Ebihara,[168] serves not only to precipitate the excess of mercury, but also to reduce any dehydroascorbic acid to ascorbic acid.

Titration. Remove 5 ml. of the filtrate with a pipet, mix with 1 ml. of *10% trichloroacetic acid*, and titrate with *standard Tillmans solution*.

NOTES. Bukin [169] replaced trichloroacetic acid and mercuric acetate, which he believes promote decomposition of the vitamin, by *Schenk reagent*, consisting of a mixture of 2% hydrochloric acid and 5% mercuric chloride,

and avoids prolonged treatment with hydrogen sulfide.

Van Eekelen [170] demonstrated that the ratio of unreduced ascorbic acid (dehydroascorbic acid) to true ascorbic acid (*l*-form) is much greater in marine plants than in marine animals as indicated by the following figures: plants unreduced 62 to 675, reduced 294 to 770 γ/g.; animals unreduced 8 to 213, reduced 41 to 687 γ/g. Various authors have reported results that indicate that the ascorbic acid of many strictly fresh fruits and vegetables, so treated as to avoid oxidation during sampling and analysis, is largely in the reduced form. The possible presence of dehydroascorbic acid should always be taken into consideration.

Fujita and Iwatake [171] consider that trichloroacetic acid is unsuited for extraction as it causes oxidation of the vitamin and decolorizes the blue solution during filtration. They and others, as noted above, substitute for it *2% metaphosphoric acid* alone or with *5% sulfuric acid*, thus obviating reduction by hydrogen sulfide.

Saha [172] and Sen-Gupta and Guha [173] suspend 10 g. of disintegrated vegetables or minced liver or fish in 50 ml. of water and run in *hydrogen sulfide* for 15 minutes. After removal of the excess hydrogen sulfide by a current of *carbon dioxide*, they add 2.5 ml. of *20% trichloroacetic acid*, centrifuge, make up to 100 ml. with water, and use an aliquot for titration.

II. Dewjatnin and Doroshenko Modification.[174] REAGENT. *Modified Standard Tillmans Reagent*, 0.001 *N*. Dissolve 0.15 to 0.20 g. of 2,6-dichlorophenol-indophenol in a small quantity of phosphate buffer at *p*H 7 and dilute to 1 liter. Standardize by mixing in a flask 10 ml. with 5 ml. of saturated sodium oxalate solution and titrating in a stream of CO_2 with 0.01 *N* $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 0.005 *N* H_2SO_4. The latter solution and the saturated sodium oxalate solution are standardized against 0.01 *N* $KMnO_4$.

The modified reagent may be more conveniently standardized against pure ascorbic acid, now readily obtainable. Keep in a dark, cool place and check its strength often.

PROCESS. *Solution.* Weigh 10 g. of the ground or pulped sample into a 200-ml. Erlenmeyer flask, add 60 ml. of *5% acetic acid*, and boil in a current of *carbon dioxide* for exactly 10 minutes from the time the first bubbles make their appearance. Cool in cold water, continuing the current of *carbon dioxide*, filter through a double thickness of gauze into a 100-ml. volumetric flask, wash with cold water up to the mark, and mix.

Defecation. Pipet 10 ml. of the filtrate into a flask, add 0.4 g. of *calcium carbonate*, shaking during the addition, then add from a buret 5 ml. of *5% lead acetate solution* and filter through a dry paper that retains well the precipitate.

Titration. Mix 10 ml. of the filtrate with 5 ml. of *80% acetic acid* in a flask and titrate in a stream of *carbon dioxide* with the *modified standard Tillmans reagent* to a pale rose color. At the beginning of the titration the solution is the color of mother-of-pearl.

Deduct from the reading that of a blank determination, using only 5 ml. of *80% acetic acid* and the *dye solution*. Complete all the operations within 30 minutes.

NOTES. Yarusova and Tomashevskaya [175] state that, although before the lead precipitation the vitamin is stable for at least an hour, after the precipitation it is unstable, especially in the presence of oxygen, hence the necessity of titrating in an atmosphere of *carbon dioxide* or *nitrogen*.

Werder and Antener,[176] in preparing the solution, reflux with 60 ml. of *5% acetic acid* for 10 minutes. If, after precipitation with the lead salt, the solution is highly colored, they remove the excess of lead with *zinc dust* and titrate with *0.005 N iodine solution in sulfuric acid* instead of with the dye solution. See Iodine Method.

Kolesnik[177] extracts the finely comminuted sample with *cold 5% acetic acid*, centrifuges instead of filters after precipitation with *lead acetate* and *calcium carbonate*, then precipitates the excess of lead with *hydrogen sulfide*, filters, and removes the excess of hydrogen sulfide by a current of *carbon dioxide*.

Iosikova,[178] in preparing the solution, first grinds 5 to 10 g. of the sample with *sand* or *powdered glass*, then with 10 to 20 ml. of *5% acetic acid*, and uses 10 to 40 ml. additional in transferring to the tube of a centrifuge. After whirling, he makes up to a definite volume in a volumetric flask and treats an aliquot with *lead acetate solution* and *dry calcium carbonate*. After centrifuging again or filtering, he makes up to a definite volume and titrates an aliquot of 10 ml. mixed with 5 ml. of *80% acetic acid*.

Folkmann Indophenol Colorimetric Method.[179] In this method, elaborated at Graz University, are combined (1) extraction with metaphosphoric acid, as first proposed by Fujita and Iwatake,[180] thus inhibiting the dehydrogenation of the ascorbic acid through the action of the enzyme ascorbinase and (2) Folkmann's novel procedure of extracting the products of the reaction by an immiscible organic solvent, thus eliminating colors and other interfering substances. Reduction by hydrogen sulfide and precipitation with mercuric acetate, mercuric chloride, or lead acetate are regarded as superfluous.

Apparatus. *Colorimeter.*

Process. *Solution.* Rub up in a mortar 10 g. of the ground or pulped material with 20 ml. of *10% metaphosphoric acid*. Mix with successive portions of water, decant after each addition onto a filter, collect the joint filtrate in a 100-ml. volumetric flask, make up to the mark, and shake.

Color Formation. Pipet an aliquot of 2, 5, or 10 ml. of the filtered solution into a 150-ml. Erlenmeyer flask, add 20 ml. of double distilled water, 1 ml. of *glacial acetic acid*, and 2 ml. of *saturated sodium oxalate solution*. Run in an excess of *0.001 N Tillmans reagent* from a buret, mix, and after allowing to stand 1 minute shake with 25 ml. of *xylene*. Allow the two layers to separate and remove the xylene solution with a pipet.

Place in a second Erlenmeyer flask 30, 27, or 22 ml. of double distilled water (corresponding respectively to 2, 5, or 10 ml. of the unknown), 2 ml. of *saturated sodium oxalate solution*, 1 ml. of *glacial acetic acid*, and an amount of *10% phosphoric acid* corresponding to that used in preparing the solution of the unknown, then add the *Tillmans reagent* and extract with *xylene* as before.

Color Comparison. Dry both xylene solutions with *anhydrous sodium sulfate* and compare in a colorimeter.

Calculation. Obtain the number of milliliters of Tillmans reagent not consumed in the reaction (K_2) by the formula:

$$K \qquad S_1 \times K_1$$

in which K_1 is the number of milliliters of Tillmans reagent taken and S_1 and S_2 are the height of the color column of the blank and the unknown respectively; then obtain the amount of Tillmans reagent consumed by subtracting K_2 from K_1.

I. Bukatsch Photometric Modification.[181] This modification differs from the original Folkmann method chiefly in that the 2,6-dichlorophenol-indophenol reagent is prepared by dissolving one of *Merck's dye tablets* in 2 ml. of water and the extinction is read in the Pulfrich photometer. In each determination, 1 ml. of the reagent equivalent to 0.5 mg. of ascorbic acid is used.

Three grams of the comminuted material and 3 ml. of *20% metaphosphoric acid solution* are ground with 27 ml. of water, allowed to stand 10 minutes, and filtered, then 5 to 10 ml. of the solution are acidified with *acetic acid* and treated with 1 ml. of the *dye solution*. The mixture is extracted with *nitrobenzene* or preferably with *xylene*, the

colors being respectively bluish red and orange. No sodium oxalate is added. Instead of making a color comparison with the blank, the color value is determined in a 0.5-cm. cell of the photometer, using filter S 50 for xylene and S 53 for nitrobenzene.

In the following calculation formula, W = milligrams of ascorbic acid in the 10-ml. aliquot equivalent to 1 g. of the sample, D = milliliters of Tillmans reagent, E = extinction reading, and X = the extinction factor calculated from the reading on the scale using standard Tillmans solution and a solution of pure ascorbic acid:

$$W = 0.5(D - XE)$$

II. Mindlin and Butler Photometric Modification.[182] Designed for blood analysis, this modification was further developed by Bessey[183] for turbid and colored plant extracts, using the Evelyn colorimeter.

III. Morell Modification. By the following simplification, Morell[184] (Bureau Plant Industry) with two assistants was able to run 120 determinations daily.

Apparatus. *Waring Blender,*[185] with 2 containers (Waring Corp., 1697 Broadway, New York).

Evelyn Photoelectric Colorimeter, equipped with green filter No. 520. Transmission limits 495 to 550 mμ.

Absorption Test Tubes, 17.5 x 2.2 cm.

Reagents. Store reagents in the refrigerator and discard after 5 days. Control the pH by the McGuinnes glass electrode.

Citrate Buffer. In 2 liters of 1.0 N NaOH dissolve 211 g. of $H_3C_6H_5O_7 \cdot H_2O$.

Citrate-Metaphosphoric Acid Buffer, pH 3.6 ($\pm$ 0.1). Mix 3200 ml. of 3% metaphosphoric acid with 868 ml. of the citrate buffer.

Modified Tillmans Reagent. Dissolve 34.4 mg. of 2,6-dichlorophenol-indophenol (Eastman Kodak Co.) in 1 liter of water.

Standard Ascorbic Acid Solution. Dissolve 25 mg. of ascorbic acid in 250 ml. of citrate-metaphosphoric acid buffer. Do not store.

Calibration of Standard Curve. Prepare in 100-ml. volumetric flasks a series containing 1 to 14 ml. of standard ascorbic acid solution in 1-ml. increments, diluted to the mark with the citrate-metaphosphoric acid buffer. Pipet 5 ml. of the dye solution into each of 14 colorimeter tubes.

Set the colorimeter in triplicate at 100% transmission, using a tube containing 5 ml. of the buffer, 5 ml. of the dye solution, and a few crystals of ascorbic acid for complete decolorization. Record the center setting, without any tube in the instrument, and maintain constant for subsequent readings.

Deliver quickly from an Ostwald pipet 5 ml. of one of the ascorbic acid solutions into one of the colorimeter tubes containing 5 ml. of the dye solution. Shake vigorously for 5 seconds and read 15 and 30 seconds after the initial mixing. Repeat for all 14 standard solutions. Take a blank reading in triplicate, using 5 ml. of buffer.

Plot a standard curve with milligrams of ascorbic acid as abscissas and log G' − log G'' as ordinates, in which G' and G'' are the galvanometer readings of the ascorbic acid and the blank respectively.

Process. *Extraction.* Place exactly 100 ml. of *3% metaphosphoric acid* in the blender, add 25 g. ($\pm$ 0.1 g.) of fresh tissue, and mix at high speed for 2 minutes. Filter about half of the suspension through a dry pleated No. 12 Whatman filter paper, discarding the first 10 ml. of cloudy filtrate, and collect about 10 ml. of the clear filtrate in a dry Erlenmeyer flask. Remove an aliquot (3 ml. for cabbage, 5 ml. for snap beans), containing 200 to 600 γ of ascorbic acid which, diluted to 50 ml., represents 4 to 12 γ/ml. To the aliquot in a 50-ml. volumetric flask, add *citrate buffer* (about 0.25 ml. for cabbage, snap beans, and several other vegetables) to pH 3.6 and dilute to volume with the *citrate-metaphosphoric acid buffer.* The final pH should be 3.6 $\pm$ 0.1. Pipet 5 ml. of the dye solution into a colorimeter tube, add 5 ml. of

the unknown solution, and proceed as in the calibration.

Galvanometer Reading. Take the readings and convert the values to gammas per milliliter, using the standard curve. In making the calculation, take account of the water in the sample.

IV. Loeffler and Ponting Modification.[186] For rapid work at the Western Regional Research Laboratory, U. S. Dept. Agr., Albany, California, the following simplification is followed.

APPARATUS. *Evelyn Photoelectric Colorimeter.*

REAGENTS. *Standard 2,6-Dichlorophenol-Indophenol Solution.* Dissolve a sufficient amount of the dye in water (about 13 γ/ml.) so that a reading (G_1) of about 30 is shown with the Evelyn instrument. For stronger solutions the reaction is not linear and a calibration curve, not a factor, must be used. Standardize by noting the reading after 15 seconds with filter No. 520, when the instrument is calibrated to 100 with water, using a tube containing 1 ml. of 1% metaphosphoric acid and 9 ml. of the dye solution.

Metaphosphoric Acid, 1%. No citrate buffer is required.

PROCESS. *Blending.* Blend 25 g. of products with high vitamin content (leaf vegetables, asparagus, raspberries, strawberries, etc.), 50 g. of those with low content (tubers, roots, stone fruits, etc.), or proportionate amounts (5 to 10 g.) of dehydrated foods, with 350 ml. of *1% metaphosphoric acid* in a machine operated for 5 minutes at high speed. Some dehydrated vegetables (sweet potatoes, carrots, etc.) may require 30 minutes of preliminary soaking in the acid; frozen foods need not be thawed. Filter through coarse pleated paper or, if starchy, through a Büchner funnel. Centrifuging is also satisfactory, since moderate turbidity is not detrimental.

Color Formation and Reading. Into each of three matched colorimeter tubes, pipet 1 ml. of the extract, add 9 ml. of water to one tube and adjust the Evelyn colorimeter to read 100; to each of the other tubes, add 9 ml. of the *dye solution* from a calibrated rapid-delivery pipet, shake slightly, place in the colorimeter, and take the reading (G_2) 15 seconds after the start of the dye addition. Obtain G_1 by reading 1 ml. of 1% metaphosphoric acid plus 9 ml. of dye solution, treated in like manner. Use filter No. 250 for all determinations.

CALCULATION. Convert G_1 and G_2 into L_1 and L_2 by means of the calibration table accompanying the Evelyn instrument and substitute in the formula $S = 10.8\ (L_1 - L_2)$ which for fruit and vegetable tissues becomes

$$T = 10.8(L_1 - L_2) \times \frac{(A + P)W}{W}$$

in which S and T are milligrams of ascorbic acid per 100 ml. of filtrate and milligrams per 100 g. of tissue respectively, A is milliliters of acid extractant, P is per cent of liquid in the sample, and W is weight of the sample.

With most fruits the volume of solids, and with dehydrated vegetables the water if below 5%, may be ignored, the formulas becoming respectively:

$$T = 10.8(L_1 - L_2) \times \frac{A + W}{W}$$

and

$$\times \frac{A}{W}$$

EXAMPLES. California berries: raspberries 25, boysenberries 15, loganberries 35, and blackberries 15 mg. per 100 g. Apples, pears, apricots, and peaches average about 5 mg. per 100 g. Morgan's figures[187] for berries (3 to 10 mg. per 100 g.) were not confirmed.

NOTE. A comparison by Ponting[188] of the efficiency of 13 acids as stabilizers for ascorbic acid, under conditions favorable to oxidation, brought out that only *metaphosphoric* and *oxalic acids* are suitable and that oxalic acid, being more stable, more easily obtaina-

ble, and less expensive, may be substituted for the metaphosphoric acid.

V. Hochberg, Melnick, and Oser Modification for Reduced and Total Ascorbic Acid.[189] The details were developed at the Food Research Laboratories, Inc., Long Island City, N. Y.

APPARATUS AND REAGENTS. See Morell Modification above.

Indophenol Reagent. Prepare a stock dye solution containing about 10 mg. of 2,6-dichlorophenol-indophenol per 500 ml. Adjust 5 ml. plus 5 ml. of citrate-metaphosphoric acid buffer (see II, above) at pH 3.5 to read exactly 0.398 (transmission = 40%) by dilution with water. Set the galvanometer at 0.0 for zero transmission and at 100.0 for a mixture of 5 ml. each of dye solution and buffer to which has been added a crystal of ascorbic for complete decolorization.

PROCESS. A. REDUCED ASCORBIC ACID. Aerate with *nitrogen* and conduct the operations under that gas.

Maceration. Treat equal weights (usually 150 g.) of the sample and *6% metaphosphoric acid* in a Waring Blendor. To secure complete inhibition of oxidases in some vegetables, add large segments directly to boiling *6% acid,* boil for 5 minutes, then cool and macerate in the blendor.

Dilution, Agitation, and Centrifuging. Draw off an aliquot of the uniform suspension, dilute with *3% metaphosphoric acid* until the solids content of the sample in suspension is not over 10%, shake mechanically for 15 minutes, and centrifuge.

Treatment with Buffer and Visual Titration. Buffer 50 ml. of the extract at pH 3.5 by adding 14 ml. of the *citrate solution,* then by means of a graduated pipet determine the approximate volume of extract required for decolorizing the 5 ml. of the *dye reagent.*

Color Formation. Dilute the volume of extract required to 10 ml. with *citrate-metaphosphoric acid buffer solution,* thus obtaining the probable optimum (2 to 6 γ/ml.) for photoelectric measurement. Place the colorimeter tube in the instrument and add 5 ml. of *dye reagent.* Fill the special pipet with the buffered extract and close the stopcock, then, with stirrer lowered, place it in the tube and open the stopcock.

Photoelectric Measurement. After 2 seconds, remove the pipet and stirrer and take the galvanometer readings 5 to 10 seconds after the opening of the stopcock.

Blank. Determine the color absorption of the reaction mixture due to foreign pigments and suspended matter, after adding a crystal of *ascorbic acid* to decolorize the dye.

CALCULATION. Construct a monogram of three parallel lines showing (a) 6 γ/ml. in tenths of ascorbic acid, (b) photometric density per 5 seconds from 0.100 to 0.400 with 0.01 divisions, and (c) same as last, but for 10 seconds.

B. TOTAL ASCORBIC ACID (MODIFIED BESSEY PROCEDURE). *Reduction.* To 25 ml. of the extract buffered at pH 3.5, add 6 drops of *caprylic alcohol* and run in a slow stream of *hydrogen sulfide* for 20 minutes. Allow to stand for 2 hours closed, then remove the excess of sulfide by running wet *nitrogen* for 2 hours through the solution.

Color Formation, Photoelectric Measurement, Blank, and *Calculation.* Proceed as under A.

EXAMPLES. The reduced, dehydro, and total ascorbic acid found by the originators were respectively: canned grapefruit juice 40, 1, and 41, and canned pineapple juice 35, 0, and 35 mg. per 100 ml.; fresh tomato juice 17, 1, and 18, fresh orange juice 49, 0, and 48, raw, fresh stringbeans 17, 6, and 23, dehydrated potatoes 0, 0, and 0, dehydrated bananas 2.5, 0.8, and 3.3, potato chips 11, 5, and 16, and milk powder 2.3, 0, and 2.1 mg. per 100 g.; pasteurized milk 0, 3.6, and 3.6, and evaporated milk 0, 0, and 0 mg. per quart.

VI. A.O.A.C. Modification. While going to press, the Association of Official Agricultural Chemists has adopted provisionally the

following reagents, standardization procedure, and process for use in the analysis of orange, grapefruit, lemon, lime, and tomato juices:

Reagents. *Acid-Stabilizing Solvent.* Dissolve with shaking 15 g. of stick glacial HPO_3 in 40 ml. of glacial acetic acid and 200 ml. of water, dilute to 500 ml., and filter rapidly into a glass-stoppered bottle. Store in a refrigerator and discard after 7 to 10 days.

Modified Tillmans Solution. Dissolve with vigorous shaking 0.05 g. of sodium 2,6-dichlorophenol-indophenol, which has been stored over soda-lime, in 10 ml. of 0.05 *M* $NaHCO_3$ plus 40 ml. of water. Dilute to 200 ml., filter through a pleated paper into an amber glass-stoppered bottle, stopper, store in a refrigerator, and protect from direct sunlight. Discard when 15 ml. of the solution, mixed with 5 ml. of the extracting solution containing an excess of ascorbic acid, is not practically colorless.

Standardize daily as follows: Place about 0.1 g. of the U.S.P. reference *l*-ascorbic acid, accurately weighed, in a 100-ml. glass-stoppered volumetric flask and dilute to the mark with the solvent. Titrate rapidly three 2-ml. aliquots, mixed with 5 ml. of the solvent in 50-ml. Erlenmeyer flasks, to a light rose-pink color which persists for at least 5 seconds. Correct for a blank titration of 7.0 ml. of the solvent plus a volume of water about equal to that of the indophenol reagent used in the direct titration.

Process. In the analysis of the unknown, 100 ml. of the juice is mixed with an equal volume of the solvent and filtered, then 10-ml. aliquots are titrated as in the standardization of the modified Tillmans solution.

Martini and Bonsignore Methylene Blue Volumetric Method.[190] It is claimed that this method is superior to the Tillmans method because of its greater specificity, sharper end-point, greater rapidity, and freedom from error due to glutathione, cysteine, and other reducing substances. It has not, however, been subjected to much critical study nor has it been so frequently modified as the older method. It is endorsed by several clinical analysts. Wachholder and Hamel[191] believe that it should replace the Tillmans method.

Reagents. *Saline Solution.* Dissolve 30 g. of sodium citrate and 8 g. of $NaHCO_3$ in 200 ml. of water.

Standard Methylene Blue Solution. Dissolve 1 part in 10,000 parts of water. Standardize against an aliquot of a solution of 4.7 mg. of pure ascorbic acid in 1000 ml. of water treated as in the actual analysis. Theoretically 1 ml. of the methylene blue solution is equivalent to 0.047 mg. of ascorbic acid. The solution keeps well.

Process. *Solution.* Triturate 0.5 g. of the sample in a mortar with *washed sand,* extract with *8% trichloroacetic acid,* and centrifuge. Decant onto a filter, collect the filtrate in a 25-ml. volumetric flask, and make up to the mark with the *trichloroacetic acid.*

Methylene Blue Decolorization. Pipet 5 ml. of the solution into a test tube, add 2 ml. of the *saline solution* and 1 ml. of *5% sodium thiosulfate solution.* Into a second test tube pipet 8 ml. of water. Add to each tube 0.2 ml. of *standard methylene blue solution* from a micro pipet and irradiate both tubes either in full sunlight or in the light of a 300-watt Philips lamp, which in the presence of ascorbic acid causes decolorization. Continue the addition of the *methylene blue solution* to the unknown until its color matches the control.

Calculation. Obtain the milligrams per gram of ascorbic acid (W) in the sample by the formula:

$$W = \frac{0.047 \times V \times 25}{0.5 \times 5} \quad 0.47V$$

in which V is the milliliters of 1 + 10,000 methylene blue solution used in the titration.

Notes. Wahren[192] uses the Pulfrich photometer.

Neuweiler[193] states that the reaction is

specific and sulfhydryl derivatives do not form colors in these concentrations. He emphasizes that the amount of ascorbic acid should be such that only 1.5 to 1.8 ml. of the methylene blue solution is reduced, that the sodium thiosulfate should not be added until the *p*H is more than 4.5 to 4.8, and that the *p*H of the methylene blue solution be kept constant within 0.05.

Mentzer and Vialard-Goudou[194] found that sulfhydryl compounds do not interfere.

Mentzer[195] reduces dehydroascorbic acid by *hydrogen sulfide* at *p*H 6.5 in a few minutes. If the *p*H is lower, the reduction is retarded and may be incomplete.

Lund and Lieck[196] note that chlorine ions and acid reaction favor the decolorization of the dye and that the observation of the color is best carried out in a bright light. In preparing the reagent, they add to 100 ml. of a *0.0015% methylene blue solution* 0.1 ml. of a solution of 9 g. of *monopotassium phosphate* and 2 g. of *sodium chloride* in 300 ml. of water and bring to a *p*H of 2 to 3. The equivalent of 0.1 ml. of the standard methylene blue solution is usually 0.002 mg. of ascorbic acid.

Roe and Hall Osazone-Furfural Colorimetric Method.[197] This method, devised at George Washington University for urine, is stated by the originators to have been widely applied to the determination of ascorbic acid in vegetable and animal tissues. The reactions involved are (1) oxidation to dehydroascorbic acid by norite, (2) formation of a crystalline osazone with dinitrophenylhydrazine, (3) reduction of nitro groups with stannous chloride and liberation of dehydroascorbic acid, (4) hydrolysis with formation of furfural, and (5) reaction of furfural with aniline acetate forming a deep red dye. The method eliminates interfering substances and is believed to be more specific than the Tillmans method.

Apparatus. *Colorimeter.*

Reagents. *Stannous Chloride Solution,* 10% in 1 + 2 HCl.

Aniline Tincture. Mix 2 volumes of redistilled aniline with 5 volumes of ethanol. Keep in a dark bottle in the refrigerator.

Dinitrophenyl-Hydrazine Reagent. Saturate 1.0 *N* HCl with 2,4-dinitrophenylhydrazine recrystallized from an ethyl acetate solution.

Standard Ascorbic Acid Solution. Dissolve 25 mg. of pure ascorbic acid in 25 ml. of 10% acetic acid containing 4% of metaphosphoric acid. Dilute 2 ml. of this solution to 100 ml. with the acetic-metaphosphoric acid solution. Add an excess of norite (5 g.), shake vigorously, and filter; 1 ml. = 0.02 mg. of ascorbic acid.

Process. *Solution.* Dissolve in 50 ml. of the vegetable or animal extract (see foregoing methods) 5 ml. of *glacial acetic acid* and 2 g. of *metaphosphoric acid,* then shake vigorously with 5 g. of *norite,* and filter. If the filtrate is not colorless, add more *norite,* shake, and filter again.

Crystallization. Prepare a series of three 15-ml. conical Pyrex centrifuge tubes, with marks at 7 ml., containing 1, 2, and 3 ml. of the norite filtrate of the sample and a second series of tubes containing 1, 2, and 3 ml. of the norite filtrate of the standard. Place the six tubes in the centrifuge and add to each at approximately the same time two volumes of *dinitrophenylhydrazine reagent* accurately measured. Allow to stand 15 to 24 hours to complete the crystallization of the osazone in the bottom of the tubes. Break up the crystals if necessary to secure packing, then centrifuge, decant carefully, set the tubes in an inverted position, and allow to drain for several minutes. Wash and thoroughly break up the mat of osazone crystals by blowing on them from a pipet 5 ml. of *10% formic acid.* Centrifuge and drain carefully.

Color Formation. Add to each tube 2 ml. of *stannous chloride solution,* place in a beaker of boiling water, and boil until dissolved, with occasional tipping of the tubes to wash down adhering crystals. Heat the tubes in

an autoclave for 30 minutes in an atmosphere of steam at 15 pounds pressure. After cooling, remove the tubes to a rack and add to each 2 ml. of *glacial acetic acid*, mix, then add 3 ml. of *aniline tincture* from the refrigerator while still cold, keeping the mixture, so far as possible, away from light after adding the aniline. Add water to the 7-ml. mark, mix by inverting, and immediately stand in cold water. After 5 minutes, remove the tubes to a rack and allow to stand 10 minutes longer.

Color Comparison. Compare the unknown with the known in a colorimeter.

CALCULATION. Calculate results by the following formula:

$$X = \frac{S}{U} \times S_1 \times \frac{1000}{N} \times 1.1$$

in which X is the milligrams of ascorbic acid per liter, S is the reading of the standard, U is the reading of the unknown, S_1 is the milligrams of ascorbic acid in the standard used, and N is the milliliters of norite filtrate.

Langou and Marenzi Phospho-Tungstic Colorimetric Method.[198] By this method both ascorbic acid and glutathione are determined in aliquots of the same solution.

APPARATUS. *Colorimeter.*

REAGENT. *Standard Cystine Solution.* Dissolve 20 mg. of cystine in 100 ml. of 0.2 N H_2SO_4.

PROCESS. *Solution.* Extract with *trichloroacetic acid* as in the Emmerie and van Eekelen Method above.

Prepare 3 tubes: In tube 1 place 2 ml. of *standard cystine solution;* in tubes 2 and 3 place aliquots of the solution of the sample containing 0.7 to 2.5 mg. of reducing constituents; add *20% sodium carbonate solution* to alkaline reaction to tube 3 and let stand 1 hour to destroy the ascorbic acid.

Reduction. To each tube add 0.2 ml. of *20% sodium sulfite solution*, allow to stand 2 minutes, then add 0.2 ml. of *20% lithium sulfate solution*, 2 ml. of *Folin phospho-tungstic reagent* (see Fugita and Ebihara Method below), and 2 ml. of *20% sodium carbonate solution.* After allowing to stand 4 minutes, dilute each tube to 25 ml. with *2% sodium sulfite solution.*

Color Comparison. Compare the color of the three solutions in the colorimeter. Tube 1 serves as the standard, tube 2 represents total glutathione plus ascorbic acid, and tube 3 represents total glutathione alone.

CALCULATION. Obtain the amount of ascorbic acid by difference, remembering that equimolar solutions of ascorbic acid and of cystine and glutathione after reduction with sodium sulfite give the same color reactions with Folin reagent.

NOTE. Adrenaline interferes by reducing Folin reagent.

Fujita and Ebihara Phospho-Tungstic Photometric Method. Fujita, Iwatake, and Miyata[199] announced that *sodium tungstate* gives with ascorbic acid a blue color reaction that is sensitive and specific and that the intensity of the color is proportional to the concentration of the vitamin, but Manceau, Policard, and Ferrand[200] claimed that the method is not specific. Later Fujita and Ebihara[201] proposed the following procedure in which the Folin phospho-tungstic reagent is used.

APPARATUS. *Pulfrich Photometer.*

A. Reduced Ascorbic Acid. REAGENTS. Keep all reagents at a temperature below 5°.

Phosphate Buffer, pH 6.2. Dissolve 8.17 g. of KH_2PO_4 and 6.97 g. of K_2HPO_4 in water and make up to 100 ml. The pH is measured electrically.

Molar Iodoacetic Acid. Dissolve 185.94 g. of crystalline monoiodoacetic acid in water, remove free iodine by adding $Na_2S_2O_3 \cdot 5H_2O$, and make up to 1 liter. Test for free iodine with starch before using.

Folin Phospho-Tungstic Reagent. Reflux for 1 hour, at first with a low flame, 17.7 g. of dry sodium tungstate (free from molybdate) with 6.5 ml. of 85% H_3PO_4 (sp.gr. 1.710) and 30 ml. of water, add a small

amount of bromine water to produce a slight color of bromine, remove the excess by boiling, cool, and dilute to 100 ml. The clear light yellow-green solution should have a pH of 3.35 measured with the quinhydrone electrode at room temperature.

PROCESS. *Solution.* Grind a suitable quantity of the sample with four times its volume of filtered *5% metaphosphoric acid* and sufficient *acid-treated and washed sand* to disintegrate the tissues; then add to the mixture five times its volume of water, mix, let stand 5 to 10 minutes, centrifuge 15 to 20 minutes at 3500 r.p.m., and filter. If the content of the vitamin is low, use half the quantities of reagents given.

Color Formation. To 4 ml. of the extract, add 2 ml. of *buffer*, heat on a water bath at 40° for 5 minutes, then add 1 ml. of *Folin phospho-tungstic reagent*, mix, and warm at 40° for 10 minutes longer. Cool under the tap for 10 minutes.

Color Reading. Conduct the reading of the rich blue liquid in the Pulfrich photometer, using a 5-mm. cell and an S 72 filter, or in a suitable colorimeter. Use water in a control determination; this is necessary for vegetable extracts, but not usually for animal extracts.

CALCULATION. Obtain the milligrams of ascorbic acid per 100 g. of the material (X) by the formula

$$X = 8. \qquad - E_0) V$$

in which E and E_0 are the extinction values in the photometer of the unknown and the blank respectively and V is the dilution (milliliters of the aliquot divided by milliliters of the total solution). If a colorimeter is used, compare with a standard solution of ascorbic acid treated in the same manner as the unknown.

B. Total Ascorbic Acid. REAGENTS. *Phosphate Buffer*, pH 1.43. Dissolve 13.62 g. of KH_2PO_4 in water, add 7.6 ml. of 85% H_3PO_4 (sp.gr. 1.710) and dilute to 100 ml.

Other Reagents as above.

PROCESS. *Solution and Defecation.* Grind a suitable quantity of the sample with 5 times its volume of *1.0 N hydrochloric acid*, 3.5 times its volume of *20% mercuric acetate solution*, and a little *sand*, then add 4 times its volume of *50% sodium acetate solution* and 0.5 times its volume of *20% lead acetate solution*, mix, centrifuge 15 to 20 minutes at 3500 r.p.m., and decant.

Reduction. Into an aliquot of the solution, in which the ascorbic acid has been converted into the dehydro form by the mercuric acetate, pass *hydrogen sulfide* for 1 hour with stirring. Stopper and keep overnight in a hydrogen sulfide atmosphere. Filter into a test tube, marking the top level, exhaust for 10 minutes to completely reduce the vitamin, and restore the volume. The pH should be 4.64 at room temperature, measured electrically.

Color Formation. To 4 ml. of the filtrate, add 2 ml. of *phosphate buffer* (pH 1.4) and 1 ml. of *monoiodoacetic acid solution* (see above), mix, and digest on a water bath for 5 minutes at 40°. Add 1 ml. of *Folin phospho-tungstic reagent* (see above), mix, warm 10 minutes on a water bath at 40° (pH 2.90 at room temperature), and cool for 10 minutes under the tap.

Color Reading. Measure the color value as above. If the solution was colored, conduct a blank determination and correct accordingly.

CALCULATION. Calculate as under A, substituting, however, the factor 8.7 for 8.13.

Various Phospho-Tungstic-Molybdic Methods. Bezssonov[202] based a test for vitamin C on the blue color formed with a modification of the Folin phospho-tungstic-molybdic reagent (not to be confused with the Folin phospho-tungstic reagent) prepared by dissolving 36 g. of *sodium tungstate* and 4 g. of *phospho-molybdic acid* in 200 ml. of water at 50°, then adding 5 ml. of *85% phosphoric acid* and 10 ml. of *sulfuric acid* drop by drop.

Salvatori [203] gives a list of other substances that react in a similar manner; nevertheless Bezssonov and van Wien [204] were able to obtain results that agreed with those by the guinea pig method. Bukin and Povolotzkaya [205] found that the reaction does not parallel anti-scurvy activity and von Euler and Burström [206] found that the results are too high.

Giri [207] uses the dark red precipitate obtained with a solution of *ammonium molybdate* as a test for ferrocyanide (Prussian blue) formed by potassium ferricyanide through the reducing power of ascorbic acid.

Bandaruk Iodate Oxidation Volumetric Method.[208] The method serves for the assay of relatively pure ascorbic acid. The reaction is as follows:

$$2C_6H_8O_6 + KIO_3 + 2HCl \rightarrow 2C_6H_6O_6 + ICl + KCl + 3H_2O$$

PROCESS. *Color Formation.* Dissolve 0.2 g. of the ascorbic acid, previously dried overnight in a sulfuric acid desiccator, in 25 ml. of *6 N hydrochloric acid* in a glass-stoppered flask, then add 10 ml. of *chloroform*.

Titration. Titrate with *standard 0.025 M potassium iodate solution* to the disappearance of the purple color. Add the last portions dropwise, stirring vigorously and continuously.

CALCULATION. Use the formula: 1 ml. of 0.025 M iodate = 0.00880 g. of ascorbic acid.

Koenig, Schiefelbusch, and Johnson Ferridipyridyl Colorimetric Method.[209] Studies made at the University of Texas brought out that ferridipyridyl sulfate, prepared by the oxidation of ferrodipyridyl sulfate with ceric sulfate, on reduction with vitamin C, forms when in excess the stable pink or deep red ferrodipyridyl ion in accordance with Beer's law. The vitamin is oxidized to threonic and oxalic acids with dehydroascorbic acid as an intermediate product.

APPARATUS. *Coleman 10–S–30 Spectrophotometer* or equivalent instrument.

REAGENTS. *Acetate Buffer.* Dissolve 60 ml. of glacial acetic acid and 10 g. of recrystallized and dried $NaC_2H_3O_2 \cdot 3H_2O$ in water, dilute to 1 liter, and adjust to pH 3.6 by adding the acid or the salt.

Ammonium Hydroxide Solution. Bubble commercial synthetic anhydrous ammonia (National Ammonia Co., Philadelphia) into water until 4 to 6 N.

Ferridipyridyl Reagent. (*a*) Prepare a stock solution of ferrodipyridyl solution as follows: dissolve 1.76 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in a little water, add 10 ml. of 1% SO_2 solution, dilute to about 800 ml., add 2.5 g. of α, α'-dipyridyl (2,2'-bipyridine), stir until dissolved, heat to boiling (or let stand overnight), cool, and dilute to 1 liter.

(*b*) Heat 200 ml. of the ferrodipyridyl stock solution to 80° and titrate with 0.08 N $Ce(SO_4)_2 \cdot 4H_2O$ solution (28 ml. of 36 N H_2SO_4, 500 ml. of water, and 40 g. of commercial $Ce(SO_4)_2 \cdot 4H_2O$, filtered after standing and diluted to 1 liter) to a change from red to yellow, about 11 ml. being required. After the sharp but slow end-point has been reached, back-titrate with 1% sulfur dioxide solution to a restored pink color. Adjust to pH 3.6 by addition of the ammonium hydroxide solution, avoiding an excess but ignoring a darkening of the solution, and dilute to 400 ml. If a precipitate forms, let settle overnight and decant. Maintain a small amount of ferrodipyridyl in the solution, if necessary, by occasional addition of sulfur dioxide. Blank determinations, made every 2 or 3 days, should be faint pink, not yellow, and stable for 2 to 3 days.

PROCESS. *Clarification.* Obtain a clear solution by filtering the solution through a Gooch crucible, by centrifuging, or by settling. Since the color when formed remains constant for several days, clarification may be carried out either before or after color formation.

Color Formation. To an aliquot, containing not more than 0.40 mg. of ascorbic acid, in a rather small volume to permit the addition of all the reagents without exceeding 100 ml. and to increase the velocity of the color reaction, add the *ammonium hydroxide solution* until Congo red paper turns the same color as in the buffer solution of pH 3.6. Maintain a pH between 2.5 and 4.0. Next add 10 ml. of the *acetic buffer* and 20 ml. of the *ferridipyridyl reagent,* heat for at least 25 minutes at 70 to 80°, or let stand 12 to 48 hours at room temperature, then cool and dilute to 100 ml.

Half or even quarter volumes of aliquot and reagents may be used for greater convenience and economy of the expensive dipyridyl. Too long heating or at too high a temperature may cause a cloudiness and require clarification.

Color Reading. When the color becomes constant, make transmittance readings in a Coleman square cuvette 1.308 cm. deep, or other suitable tube or cell, at 28 to 32°, with two successive portions at 510-mμ wave length. Use as a reference solution a blank containing 5 ml. of buffer and 10 ml. of the reagent per 50 ml. Derive the result from a previously prepared graph.

If desired, the color intensity may be compared in a colorimeter with a solution containing known amounts of the vitamin treated in the same manner as the unknown.

Kirk Polarographic Method.[210] *Preliminary.* The results of preliminary vitamin C determinations, made at the New York Agricultural Experiment Station, using a Fisher electropode or polarograph, indicate that the method can be adapted for quantitative work.

Other Methods. Tauber and Kleiner[211] follow in essential details the *mercuric acetate defecation process* of Emmerie and van Eekelen; they then mix 5 ml. of the extract with 2 ml. of *0.4% potassium ferricyanide solution,* heat at 40° for 3 minutes, cool, and add 5 ml. of *Folin and Malmros ferric gum ghatti solution.* Color comparison is made with a standard prepared from 1 ml. of a *0.025% solution of ascorbic acid* in *0.01 N hydrochloric acid* containing *0.05% of cystine* treated like the known.[212] They report the reducing power of tissue extracts before and after the action of *ascorbinase,* obtained from both the summer and Hubbard squash. Further steps, including defecation with *mercuric acetate* and color formation with *Tillmans reagent,* are similar to those of the Emmerie and van Eekelen method.

Spruyt and Donath[213] and Bezssonoff and Vertruyen[214] also employ *ascorbinase,* the former from the kelor (*Moringa pterygosperma*) and the latter from the pumpkin.

Meunier[215] and Meunier and Mentzer[216] employ *phosphoric* and *trichloroacetic acids* in a kinetic method featuring the electrophotometer.

Harde[217] obtained by reduction of *silver nitrate solution* figures that paralleled those by the Tillmans method.

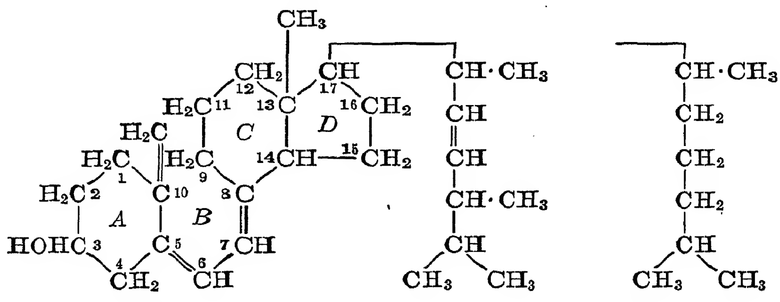
A recent and unique method, described by Espil and Genevois,[218] involves precipitation with *dinitrophenylhydrazine* and titration of the precipitate with *0.002 N titanium trichloride in 2 N hydrochloric acid,* using *methylene blue indicator.*

Vitamin D

A number of natural and activated sterols have pronounced antirachitic properties, owing to their action in promoting calcium and phosphorus assimilation. Fish liver oils are rich in vitamin D and from these are prepared concentrates; milk, butter, egg yolk, and liver are natural sources of the vitamin. Vegetable foods contain little or none.

Vitamin D_1, once thought to be the pure vitamin, is now considered to be calciferol plus lumisterol. D_2 is calciferol (irradiated ergosterol) and D_3 is the natural vitamin which is prepared artificially by irradiating

dehydrocholesterol. The formula for vitamin D_3 differs from that of vitamin D_2 in the structure of the side chain as shown below.



VITAMIN D_2 OR CALCIFEROL
(Heilbron *et al.*)

SUGAR CHAIN OF
VITAMIN D_3

Both D_2 and D_3 are white, dextrorotatory (+102.5° and +83.3°) powders, soluble in ethanol and fat solvents but not in water.

Analytical Methods. **Brockmann and Chen Antimony Trichloride Colorimetric Method.**[219] The color formed with antimony trichloride in the presence of calciferol (D_2), natural vitamin D (D_3), and tachysterol, variously described as orange-yellow or pink, is radically different from the blue formed by the same reagent with vitamin A in the Carr and Price method. The originators (Göttingen University) of the method claim that vitamin A and sterols other than tachysterol do not interfere, presumably if the observations are made in a comparison spectroscope; Milas, Heggie, and Raynolds,[220] however, found that in the presence of vitamin A the results, following a modified procedure and using a Hardy color analyzer, require correction. Ritsert [221] states that the method is not suited for irradiated yeast and products containing ergosterol, 7-dehydrocholesterol, and other provitamins, for fish liver oils containing vitamin A, or for biological solutions with low vitamin D concentration, but partial removal of interfering substances may be effected by adsorption on aluminum oxide from a methanol-benzene-benzine solution.

APPARATUS. *Hellige Comparator.*

Comparison Glass Prism Spectroscope, showing absorption bands of the visual spectrum.

REAGENT. *Antimony Trichloride Solution,* 30% in purified, dried, ethanol-free $CHCl_3$.

STANDARD GRAPH. Prepare a series of standard solutions of 0.2 ml. each, containing 0.02 to 0.4 mg. of *crystalline calciferol* dissolved in pure dry *chloroform.* To each solution add 4 ml. of cold saturated solution of *antimony trichloride in chloroform.* In the absence of vitamin A, allow to react exactly 10 minutes, transfer each solution to one of the cells of a Hellige comparator, or other type of colorimeter, and adjust the thickness of the layer so that the color is just visible.

In the presence of vitamin A, employ a comparison spectrum limiting the portion observed to a narrow region extending slightly beyond the absorption band on each side and maintaining a uniform width of slit in all observations.

Plot a curve with milligrams of the vitamin as abscissas and extinction readings (E) as ordinates.

PROCESS. *Solution and Color Formation.* Tare a melting-point tube on a micro bal-

ance, place in the tube a quantity of a solid alcohol-free substance containing 0.04 to 0.4 mg. of vitamin D or, if an oil, place an equivalent amount on the tube, and carefully weigh. Dissolve the weighed substance in 0.2 ml. of strictly dry *chloroform* with the aid of heat and add 4 ml. of *antimony trichloride solution.*

Reading. Proceed as with the standard.

CALCULATION. Compare the reading with the standard graph.

I. Raoul and Meunier Acetic Anhydride Photometric Modification.[222] By the use of a combination of the Carr and Price and the Liebermann reagents, the interference of sterols with transconfiguration is eliminated and sterols with biological activity are differentiated from each other and from vitamin A.

APPARATUS. *Electrophotometer*, with blue color filter No. 30.

...NT. Saturate 30 ml. of $CHCl_3$ with ...$_3$, then add 3 ml. of acetic anhydride and 5 drops of H_2SO_4.

PROCESS. Pipet into the colorimeter cell 3.5 ml. of the freshly prepared *reagent* and 0.5 ml. of a chloroform solution of the sterol, containing preferably 50 γ, and determine at 30-second intervals the color value, using blue color filter No. 30.

Vitamins D_2 and D_3, with three double bonds, produce an intense blue color that fades rapidly, whereas ergosterol (provitamin D_2), lumisterol and 7,8-dehydrocholesterol (provitamin D_3) with two conjugated double bonds produce a blue color that remains constant, and cholesterol with one double bond reacts very much more slowly.

II. Nield, Russell, and Zimmerli Acetyl Chloride Photometric Modification.[223] These investigators (New Jersey Experiment Station) by substituting acetyl chloride for hydrogen chloride and acetic anhydride, prepare an antimony trichloride reagent that is stable at least 9 weeks and is three times as sensitive as that of Brockmann and Chen. They have applied the method to the pure vitamins only, but propose to adapt the method for poultry feed, etc.

APPARATUS. *Bausch & Lomb Universal Spectrophotometer*, with standard cell capacity of slighty over 23 ml.

REAGENTS. *Chloroform*, purified. Wash Merck's reagent chloroform seven times with equal portions of distilled water, shake with an excess of P_2O_5, and run rapidly through a filter paper; fractionate and discard the first cloudy portions and the last 10 ml.

Antimony Trichloride Reagent. Far greater care must be taken in the preparation of this reagent than for that for the determination of vitamin A. Dissolve 15 to 22 g. of Merck's reagent $SbCl_3$ in 100 ml. of purified $CHCl_3$ and warm to 35 to 45° to effect rapid solution. Filter and add to every 100 ml. of filtrate 2.0 ml. of Merck's redistilled acetyl chloride.

PROCESS. *Solution.* Dissolve the vitamin in *purified chloroform.*

Color Formation. Run from a micro buret 0.10 to 1.00 ml. of the solution, containing 2 to 20 γ of the vitamin, into a glass-stoppered graduated cylinder and make up to 25 ml. with *antimony trichloride reagent.*

Color Reading. Eliminate the blank cell and determine the optical density of the yellow-pink solution at 500 and 550 mμ within 4 minutes in the spectrophotometer. The difference between the two represents the absorption due to the reaction product of the vitamin plus the reagent, thus obviating errors which might arise from differences in general absorption by the blank cell and that containing the test solution.

CALCULATION. The optical density of the colored solution is proportional to the vitamin concentration. The $E^{1\%}_{1\text{ cm.}}$ values for crystalline D_2 and D_3, calculated from density readings at 500 mμ for 8 γ of vitamin D in 25 ml. of solution are of the same magnitude, namely, approximately 1800.

III. Milas, Heggie, and Raynolds Maleic Anhydride Photometric Modification.[224] In

the examination of high potency fish liver oils, Milas, Heggie, and Raynolds (Massachusetts Institute of Technology), by a modified Brockmann and Chen antimony trichloride method, obtained results agreeing fairly well with those of the biological method, provided corrections are introduced for the disturbing influence of vitamin A, carotenoids, and 7-dehydrocholesterol. Since the modification is cumbersome, not of universal application, and employs a color analyzer costing between five and six thousand dollars, a procedure is proposed by which vitamin A and carotenoids, and also possibly cholesterol, are destroyed by treatment of the unsaponifiable matter with maleic anhydride, then antimony trichloride is added and the color transmission (or absorption) in the visible region of the spectrum is determined.

Apparatus. *Spectrophotometer*, or a photometer provided with a suitable color filter.

Reagent. *Maleic Anhydride.* The reagent is most effective if freshly prepared by distillation in a moderate vacuum of 1 part of maleic acid and 1.5 parts of P_2O_5.

Process. *Separation of Unsaponifiable Matter.* Saponify about 1 g. of the oil, separate the unsaponifiable matter, and dry by dissolving in *absolute ethanol-benzene mixture* and distilling under reduced pressure.

Removal of Sterols. To remove the greater portion of the sterols, dissolve the dry residue in about 5 ml. of *pure methanol*, cool to −10 to −15°, and filter at the same temperature. Completely remove the methanol from the filtrate by distillation and drying.

Destruction of Vitamin A and Carotenoids. Dissolve the residue in about 5 ml. of freshly purified peroxide-free *dioxane* and add about 0.2 g. of *maleic anhydride* in about 2 ml. of *1,4-dioxane*. Heat on the water bath for 1 hour, cool, add 10 ml. of *ethanolic 0.5 N potassium hydroxide solution*, and allow to stand 5 to 10 minutes at room temperature, then add 15 ml. of water.

Ether Extraction. Shake the aqueous solution with several portions of *ether*, wash the combined ether solutions with 2 portions of water, dry over *anhydrous sodium sulfate*, filter, and distil the ether from the filtrate under reduced pressure.

Color Formation and Reading. Dissolve in a 1-cm. special spectroscopic cell the residue in 0.2 ml. of strictly dry *chloroform*, add 3.8 ml. of *saturated antimony trichloride solution in strictly pure dry chloroform*, and allow to react 10 minutes. Read the per cent of transmission in a photometer provided with a filter excluding all rays but the band at about 500 to 520 mμ or in a spectrophotometer set for wave length 500 mμ.

Calculation. Calculate the $E^{1\%}_{1\,\text{cm.}}$ value by the formula

. 1

in which T is the per cent of transmission and c is the per cent of concentration.

Notes. Milas and associates have performed a real service in breaking away from adherence to standard potency oils or concentrates and adopting purified crystalline calciferol (vitamin D_2) as the standard. The E value at 500 mμ of a 1% solution of the calciferol in a 1-cm. cell was 890 and the potency as determined by R. S. Harris was 40,000,000 U.S.P. units per gram. Calculated from these data, the value E represents 0.000,001,123 g. = 1123.6 γ of calciferol or, expressed in terms of potency, 44,944 units, that is, forty times the gammas of calciferol. These factors permit calculation of results in terms of gammas or U.S.P. units per gram or milliliter.

Nakamiya and Koizumi,[225] in a preliminary treatment, destroy vitamin A by irradiation with the rays from a mercury lamp. Vitamin D is also destroyed to some extent by the irradiation, but much more slowly; hence, by limiting the time of action, fairly accurate results are obtained.

IV. Ewing, Kingsley, Brown, and Emmett Chromatographic Modification.[226] Although designed for fish liver oils, the procedure is applicable to other oils. The first two authors are at the Michigan State College, the second two are with Parke, Davis and Company.

Apparatus. *Bausch & Lomb Visual Spectrophotometer*, equipped with Martins polarizer and 1- or 2-cm. cells.

Reagents. *Superfiltrol* (The Filtrol Corp., Los Angeles). Pack in two portions in a tube, 7 mm. in diameter, over a wad of cotton and press firmly with a cork-end rod under 6 cm. suction to the heights indicated below.

Ethanolic Potassium Hydroxide Solution. Dissolve 14 g. of pellets in ethanol and dilute to 500 ml. Protect from CO_2 and filter on hardened paper as needed.

Ether, anhydrous. Wash c.p. ether with 1% $FeSO_4 \cdot 7H_2O$ solution to remove peroxides, then ten times with water to remove ethanol, dry with P_2O_5, filter, and store over sodium. Distil from metallic sodium as needed.

Naphtha. Shake Skellysolve with H_2SO_4, wash with 10% Na_2CO_3 solution, first twice alone, then with addition of 5% $KMnO_4$, and finally fifteen times with water. Dry over sodium and distil at 68 to 70°, rejecting the first 5% and last 10% of distillate.

Chloroform. Wash $CHCl_3$ well with seven equal portions of water, dry over anhydrous potassium carbonate, decant, and distil, rejecting the first and last 10%. Prepare as needed in small lots, test with silver nitrate solution for chlorine and with starch-potassium iodide solution for oxidizing agents, then, just before using, shake with activated carbon and filter. Prepare weekly and protect from light.

Antimony Trichloride Reagent. Dissolve 18 g. of the salt in purified $CHCl_3$, dilute to 100 ml., filter, and add 2 ml. of redistilled acetyl chloride. Prepare weekly.

Benzene. Dry c.p. thiophene-free benzene over sodium, distil, and shake with Superfiltrol as needed.

Process. *Saponification.* Weigh 0.5 to 2 g. of natural oils, or 0.1 g. of concentrates, into a 125-ml. Erlenmeyer flask, add 10 ml. of *ethanolic potassium hydroxide solution* (1 ml. per gram of charge if over 1 g.), place a short-stemmed funnel in the neck of the flask, and heat for 1 hour or more with shaking in a water bath at 70 to 75°.

Ether Extraction. Cool to room temperature, add 20 ml. of water per 10 ml. of the alkali solution, and extract in a separatory funnel with four 25-ml. portions of *ether* with gentle shaking, breaking up any emulsion formed with a few drops of *ethanol.* Wash the combined clear ether extracts in the separatory funnel with three 50-ml. portions of water, adding 2 ml. of *ethanol* if an emulsion forms. Finally wash with four 25-ml. portions of water with vigorous shaking, removing the aqueous layer when clear, but continuing the treatment if *phenolphthalein* gives a pink color after the third washing. Draw off the washed ether extract into a 125-ml. Erlenmeyer flask through a funnel containing *anhydrous sodium sulfate,* rinsing with 25 ml. of *ether.* Evaporate the ether under reduced pressure in a water bath at 50°. Take up the residue in 5 ml. of a *mixed solvent* (50 parts by volume of naphtha, 10 parts of anhydrous ether, and 1 part of absolute ethanol) and add 1 drop of *Sudan III solution* (25 mg. in 1 liter of the mixed solvent).

Chromatographing the Mixed Solvent Solution. Wet a 6-cm. Superfiltrol adsorption column with 10 ml. of the *mixed solvent,* add the solution of the sample, and rinse with 5 ml. of the *mixed solvent*, then add 35 ml. of the *mixed solvent*, each addition being made just before the top of the column becomes dry, using a pressure of 6 cm. of mercury increased, after the last addition, to 10 cm. Dry by drawing air through the column for 5 to 10 minutes.

Separation of Vitamin A plus Pigments.

Remove with an L-bent spatula the top layer of the column to a point 2 mm. below the red Sudan III band.

Elution of Vitamin D and Sterols. Treat the remainder of the column with 25 ml. of *ether*, using suction. Evaporate to dryness under reduced pressure the combined filtrate and eluate, then take up in 10 ml. of *purified chloroform.*

Color Formation and Extinction Reading. (*a*) *Vitamin D and Sterols.* Add to 1 ml. of the chloroform solution 10 ml. of *antimony trichloride reagent,* swirl for 30 seconds, fill the 2-cm. absorption cell, and determine the extinction at 500 mμ exactly 3 minutes after adding the reagent.

(*b*) *Sterols.* Evaporate another 1-ml. aliquot of the chloroform solution to dryness and take up in 5 ml. of *1 + 2 naphtha-benzene mixture.* Wet a tightly packed 1.5-cm. Superfiltrol column with 5 ml. of the *mixed solvent,* add the naphtha-benzene solution of sterols, then 5 ml. of the *mixed solvent* to rinse the flask and 50 ml. to elute the sterols. Rejecting vitamin D in the lavender-blue band in the upper portion of the column, evaporate the filtrate under reduced pressure and take up the residue, consisting of sterols, in 1 ml. of *chloroform.* Add to this 10 ml. of *antimony trichloride reagent* and determine the extinction in a 2-cm. cell at 500 mμ exactly 3 minutes after mixing.

CALCULATION. From the two extinction values, calculate $E^{1\%}_{1\,\text{cm.}}$ for vitamin D plus sterols and for sterols alone, then by difference for vitamin D alone. To obtain the potency in U.S.P. units per gram of oil, multiply the latter value by the conversion factor 19,300 which is 15,000 (the average potency of a typical vitamin D mixed fish liver oil) divided by 0.778 (the average $E^{1\%}_{1\,\text{cm.}}$, 500 mμ of that oil).

EXAMPLES. Ewing *et al.* report (1) $E^{1\%}_{1\,\text{cm.}}$ 500 mμ, (2) calculated U.S.P. units per gram, and (3) Biological Method, U.S.P. units per gram respectively as follows: low vitamin D fish liver oil blend 0.14 to 0.43, 2700 to 8300, and 3000 to 6600; high vitamin D fish liver oil blend 0.65 to 1.89, 12,500 to 36,500, and 12,000 to 35,000; tuna liver oils 0.59 to 1.40, 11,400 to 27,000, and 12,000 to 28,000; cod liver oil 0.008 to 0.0165, 154 to 318, and 250 to 300; halibut liver oil 0.066 to 0.099, 1270 to 1910, and 1200 to 1200; yellow fin liver oil 0.153 to 0.162, 2950 to 3130, and 3000; swordfish liver oil 0.34 to 0.48, 6560 to 9260, and 5000 to 10,000; albacore liver oil 2.94 to 3.03, 56,700 to 58,500, and 55,000; bonita liver oil 3.11 to 3.33, 60,000 to 64,300, and 65,000.

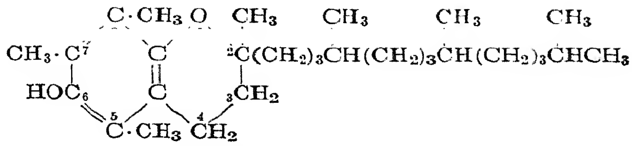
V. Gudlet Modification. Gudlet[227] observes that colorimetric methods that are suited for the pure substance or high concentrates are not satisfactory for low concentrations such as fish oils because of the interference of saponifiable oil, sterols, and vitamin A. He saponifies the oil, separates the unsaponifiable fraction, freezes out the sterols by the Windaus method, and removes vitamin A by condensing with maleic anhydride by the Dalmer method, then determines vitamin D by the antimony trichloride method, using the Pulfrich photometer as directed by Brodmann.

Aronov and Geshelina,[228] in applying the Gudlet procedure, found that it is essential to stir vigorously the mixture during saponification, use a considerable amount of *absolute methanol* for dissolving the unsaponifiable fraction, and add *maleic anhydride* in excess for the removal of vitamin A, as well as to operate on a liberal charge.

TOCOPHEROL

(Vitamin E)

Three anti-sterility vitamins (α-, β-, and γ-tocopherol) occur in nature. Cereal germs are ideal sources; legumes, cottonseed, leaf vegetables, milk, eggs, and meat also contain small amounts.



α-Tocopherol (Fernholz)

The tocopherols are yellowish oils, soluble in fat solvents but not in water.

Analytical Methods. See also Part II, G7. **Emmerie and Engel Ferric Chloride-Dipyridyl Colorimetric Method.**[229] The method (Utrecht University) is based on the reducing properties of tocopherol toward ferric chloride and the color of the ferrous salt thus formed, in the presence of dipyridyl. It is stated to be simpler, more rapid, and more sensitive than potentiometric titration with auric chloride. By adsorption, carotene which also reduces ferric chloride, is removed[230] and, by reduction of the amount of potassium hydroxide solutions used to the minimum quantity required for saponification, the destructive action of the alkali is controlled.[231] For wheat germ oil, olive oil, and doubtless other oils, the details are as follows.

Apparatus. *Pulfrich Photometer* (Zeiss), provided with filter No. 50 and 1-cm. cell, or other form of photometer or spectrophotometer.

Reagents. *Ferric Chloride Solution*, 0.2% in absolute ethanol. Prepare freshly from $FeCl_3 \cdot 6H_2O$.

α : α′-Dipyridyl Solution, 0.5% in absolute ethanol. The solution must be freshly prepared.

Floridin XS Earth Column. Fill a 12 x 30 mm. tube with the earth purified as follows. Digest a suitable quantity on a boiling water bath for 1 hour with HCl, repeating at room temperature with several fresh portions, and wash with water until the acid is removed, then with ethanol, and finally with benzene. Dry at room temperature.

Methanolic Potassium Hydroxide Solution, 2 *N*. Dissolve 112 g. of KOH in methanol and make up to 1 liter with the solvent.

Process. *Saponification.* Saponify 1 g. of the oil in a test tube (16 x 140 mm.) attached to a reflux condenser with 2 ml. of *2 N methanolic potassium hydroxide solution* for 10 minutes at 72 to 74° in an atmosphere of *nitrogen*. Dilute with 8 ml. of *methanol* and 10 ml. of water, and extract three times with 50 ml. of *peroxide-free ether*. Wash the combined ether extracts with water, *2% aqueous potassium hydroxide solution,* and finally again with water until free from alkali. Dry the extract (unsaponifiable matter) over *anhydrous sodium sulfate* and evaporate *in vacuo* in an atmosphere of *carbon dioxide*.

If carotene is not present, dissolve the residue in 25 ml. of *ethanol*.

Removal of Carotene. If carotene is present, dissolve the reside in 5 ml. of *benzene* and pass through a *Floridin XS column* previously wet with *benzene,* then wash out with *benzene* until the total eluate amounts to 25 ml. Carotenoids and vitamin A (limit 320 I.U.) color the clay a greenish blue and dark blue respectively.

Reduction. To 1 ml. (or more) of the solution of the unsaponifiable matter containing 0.1 to 0.4 mg. of tocopherol, add 1 ml. of *0.2% ferric chloride solution,* mix, then add 1 ml. of the *dipyridyl solution,* and make up to 25 ml. Prepare in like manner a blank solution.

Color Reading. After allowing to stand 10 to 15 minutes, compare the solution in the photometer with a standard solution prepared from pure tocopherol treated with the same amounts of the reagents.

Calculation. Correct known and un-

known for the blank determination. Calculate the result in gammas per gram or milliliter of the sample by rule of three.

NOTE. The same authors [232] also studied the absorptive action of Merck's alumina, standardized by the Brockmann method, on carotenoids, vitamin A, and tocopherol.

Meunier and Vinet Prussian Blue Colorimetric Method.[233] The method is a generic one for 1,2- or 1,4-diphenols.

APPARATUS. *Photoelectric Colorimeter.*

REAGENT. *Ferric Chloride Reagent.* To 1 drop of 1% $K_3Fe(CN)_6$ solution, add 1 drop of 1.5% $FeCl_3 \cdot 6H_2O$ solution, mix, and dilute with 10 ml. of ethanol free from reducing substances. The solution is clear and of a mahogany color. Use at once or store in the dark.

PROCESS. In the determination of tocopherol, the reaction is applicable directly to vegetable oils, if free from carotene. Vitamin A, as well as carotenoids (in butter), must be removed from animal organs. The reduction of the ferric chloride in neutral solution takes place in 5 seconds, 1 molecule of the vitamin reacting with 2 molecules of ferric chloride to form a quinone and Prussian blue.

Karrer, Jaeger, and Keller Auric Chloride Potentiometric Method. Karrer, Escher, Fritsche, Keller, Ringier, and Salomon [234] studied the potentiometric titration of α-tocopherol with silver nitrate and auric chloride solutions and proposed the latter as a method for the determination of the vitamin in natural products. Strongly reducing cell constituents (glutathione, ascorbic acid, etc.) do not interfere. The dehydrogenated product is a yellow oil ($C_{29}H_{50}O_3$) to which has been assigned a structural formula with a break in the ring between 3 and 4 and a hydroxyl group at 3 in accord with Karrer and Keller's coumarane formula for the vitamin. The details of the method, as applied to vegetable products (wheat and maize germ, lettuce, and seed oils), were elaborated by Karrer and Keller [235] and as applied to animal products by Karrer, Jaeger, and Keller.[236]

Karrer and Keller [235] also Karrer and Jaeger [237] note that the "carotenoid error" must be considered. Titrated in ethanol, β-carotene, α-carotene, lycopene, xanthophyl, and zeaxanthin require 8 equivalents of auric chloride and astacin and rhodoxanthin 2 equivalents, but the conditions are so adjusted that crocetin, bixin, fucoxanthin, and violaxanthin are not oxidized by auric chloride.

APPARATUS. *A pH Electrometer*, equipped with a platinum or gold electrode for redox measurements. Suitable instruments are supplied by the Coleman Electric Company and other instrument makers.

REAGENTS. *Auric Chloride Solution*, 0.01 *N.*

Antimony Trichloride Solution in Chloroform. See Carr and Price Method for Vitamin A above.

PROCESS. *Extraction.* (*a*) *Vegetable Products.* Dry vegetables and other succulent foods at a moderate heat, then grind and extract 1 to 10 kilos of the air-dry material with *benzene*. Dissolve 2 to 3 g. of a germ oil in 50 to 80 ml. of *benzene* and pour into 250 ml. of *ethanol*. The extract contains, in addition to fat and tocopherol, vitamins A and D. Distil off the benzene from the extract and dry. Weigh if desired.

(*b*) *Animal Products.* Digest the finely chopped meat or organs twice with 4 parts of *ethanol*, the first for 3 hours, the second overnight. Press the liquid out of the residue and extract twice with 4 parts of *1 + 1 ethanol-benzene mixture* as described for the ethanol extraction. Mix the four extracts and evaporate in a stream of *nitrogen in vacuo* until the solvents are completely removed. Weigh if desired.

Saponification. Saponify the residue (*a* or *b*) by heating on a boiling water bath for 2 hours with 250 ml. (or more if necessary) of

10% methanolic potassium hydroxide solution in a stream of *nitrogen gas*. Saponify 2 g. of oils and fats in the same manner, but the residue from lard and some other animal fats may contain undecomposed glycerides, thus necessitating a second saponification.

Dilute the saponified mass with 4 volumes of water and extract by shaking with several portions of *ether* in the usual manner. Wash the ethereal solution with several portions of water until neutral. Separate the ethereal solution and dry with *anhydrous sodium sulfate*. Distil off the bulk of the ether in a stream of *nitrogen*, transfer the residue to a dish, and heat further to remove the remainder of the ether. Weigh the unsaponifiable matter if desired.

Removal of Sterols. Boil the unsaponifiable matter with 500 ml. of *ethanol*, cool in a refrigerator overnight, and pipet off the ethanol solution from the sterol crystals. Repeat the operations and distil off the ethanol from the combined solution, then heat further to remove the remainder of the solvent.

Removal of Vitamin A. Dissolve the unsaponifiable matter in *chloroform*, add an excess of *antimony trichloride solution in chloroform*, and allow to stand for 30 minutes. Shake the chloroform layer with *1 + 1 hydrochloric acid* to dissolve the antimony compounds and wash until neutral. Shake out the acid wash liquid with *chloroform* to recover any vitamin that passed into solution, and add this chloroform extract to the main chloroform extract. Distil off the chloroform, dry, and, if desired, weigh.

Preparation of Solution for Titration. Dissolve 100 mg. of the unsaponifiable matter, freed from sterols and vitamin A if necessary, in 50 ml. of *80% ethanol*, taking care that the solution is complete. Remove with a pipet 25 ml. of the solution, dilute with 250 to 400 ml. of *80% ethanol*, and heat at 50° to insure complete solution.

For direct titration of vegetable oils, which yields good results, dissolve 2 to 3 g. of the sample in 50 to 80 ml. of *benzene*, pour the solution into 250 to 350 ml. of *strong ethanol* (not 80%), and add a little *lithium chloride* to increase the conductivity.

Auric Chloride Titration. Introduce the solution into an electrometer cell, add *0.01 N auric chloride solution* in 0.5- to 1.0-ml. portions up to 4.0 to 5.0 ml., and take the potentiometric difference readings (E) in millivolts after each addition.

CALCULATION. Plot a curve with 0.0 to 5.0 millivolts (E) as ordinates and 1.0 to 5.0 milliliters of 0.01 N auric chloride as abscissas; note the sudden jump from a curve proper to a straight line and record the milliliters of 0.01 N auric chloride solution (G) corresponding to the middle of this line.

Obtain the milligrams of tocopherol (T) by the formula

$$T = \frac{430}{2} \times \frac{G}{100} = 2.15G$$

in which 430 is the molecular weight of tocopherol, 2 is the equivalent of auric chloride that reacts with 1 equivalent of tocopherol (3 moles of tocopherol require 2 moles of $AuCl_3$).

Correction for Carotene. If carotene is present (only in liver of animal products), acetylate one-third of the unsaponifiable matter by heating 2 hours on a water bath with an equal volume of *acetic anhydride in pyridine*. Shake with *naphtha*, treat the naphtha extract with *5% hydrochloric acid*, draw off the acid layer, and wash the extract with water until neutral. Distil off the naphtha, remove the residue to a dish, dry, weigh, and titrate with *auric chloride solution*. Deduct the middle reading from the middle reading made on the unacetylated portion.

NOTE. Karrer, Jaeger, and Keller [238] give the following results on α-tocopherol in animal products by the potentiometric and by the Emmerie and Engel colorimetric methods respectively: beef muscle 5.9 and 6.2, horse

muscle 5.3 and . . ., beef liver 9.5 and 10.6, horse liver 13.2 and 14.9, horse heart 4.9 and 6.2, horse kidney 6.3 and . . ., and lard 2.2 and 2.0 γ/g. In vegetable products Karrer and Keller[235] found by the potentiometric method: wheat germ oil 52.0, maize germ oil 43.0, dried lettuce 550, linseed oil 230, olive oil 80, sesame oil 50, and palm oil 27 γ/g.

Smith, Kolthoff, and Spillane Auric Chloride Amperometric Method.[239] Recognizing that the polarographic method is complicated and subject to an error of 3 to 5% in 0.001 *M* solutions, Smith and his collaborators at the University of Minnesota turned to amperometric titration with the dropping mercury electrode as indicator.

Apparatus. *Cell and Equipment.*[240] Provision is made for removal of the mercury dropping from the electrode, thus avoiding its reaction with an excess of auric chloride. The capillary has the following characteristics: In 0.1 *M* potassium nitrate, containing 0.001 *M* nitric acid, and under 62.7 cm. mercury pressure, t = 3.72 sec. (open circuit), m = 1.52 mg. sec.$^{-1}$, and hence $m^{2/3}t^{1/6}$ = 1.646.

Reagents. *Standard Chloroauric Acid* ($HAuCl_4 \cdot 3H_2O$), 0.00979 *M* in 75% ethanol. Standardize with either ferrous sulfate or oxalic acid as reducing agent as described in standard works on analytical chemistry.

Buffer. Benzoic acid 0.1 *M*, sodium benzoate 0.1 *M*, and NaCl 0.1 *M* in 75% ethanol.

Process. *Preliminary.* Dissolve a weighed charge of the tocopherol in a small amount of *95% ethanol*, transfer to the electrolysis cell, rinse with a solution of the supporting *electrolyte in 75% ethanol*, and add a further amount to a total of 90 ml. Place the cell in a thermostat and pass *nitrogen gas* through the solution for 20 to 25 minutes to remove dissolved oxygen.

Titration. Connect by means of a salt bridge with a saturated calomel electrode and titrate at the desired applied potential, adding the *standard chloroauric acid solution* in portions from a buret and passing a current of *nitrogen* for 1 to 2 minutes through the mixture in the cell after each addition.

Accuracy. In a concentration range between 1×10^{-3} and 3×10^{-4} *M*, the accuracy is 0.3%.

Furter and Meyer Nitric Acid Photometric Method.[241] These authors (Zürich and Basel) found that an intense cinnabar red coloration results from heating tocopherol dissolved in absolute ethanol with nitric acid. The colorless solution first changes to yellow, then to orange-red, and finally to the characteristic color on which Furter and Meyer base their method.

Dry powdered foods are extracted with absolute ethanol and the fluid extract is heated with the nitric acid, whereas oils are heated directly with absolute ethanol and the acid.

Apparatus. *Microcohobator.* The apparatus is preferably all glass, but a 25-ml. flask fitted with a perforated, well-paraffined stopper and a tube 8 mm. wide and 50 cm. long serving as a condenser answers the purpose. See Furter.[242]

Pulfrich Photometer, provided with color filter No. 47.

Process. A. Dry Powdered Foods. *Extraction.* Weigh into a 100-ml. graduated flask 10 g. of a ground cereal, oil seed, or leguminous product, or a smaller amount of a tocopherol-rich material such as germ meal. Add 75 ml. of *absolute ethanol*, reflux for 6 minutes, cool, make up to the mark with *absolute ethanol*, and filter. Concentrate or dilute with *absolute ethanol* an aliquot of the filtrate so that 5 ml. will contain about 0.3 mg. of tocopherol and pipet 5 ml. of the adjusted extract into a 25-ml. flask.

Color Formation. To the extract, add 1 ml. of *nitric acid* from a buret with continual rotation and a small piece of *pumice stone*, then reflux gently for 3 minutes, avoiding concentration. Cover the flask, cool for 15 minutes, and transfer by means of a pipet to a

1-ml. cell of the photometer an amount of the acid mixture sufficient for observation. In order to compensate for any absorption by the solvent, place in the other 1-ml. cell a mixture of 5 ml. of *absolute ethanol* and 1 ml. of *nitric acid*.

Color Reading and Calculation. Obtain the extinction reading (E), using filter No. 47, and compare with the straight line in a graph prepared with 0 to 1.0 mg. of tocopherol with 0.2-mg. intervals as abscissas and 0 to $2.0E$ with $0.5E$ intervals as ordinates.

B. Oils. *Color Formation.* Heat for 5 minutes with continuous rotation 0.2 to 0.3 g. of the oil in a 25-ml. flask with 5 ml. of *absolute ethanol* and 1 ml. of *nitric acid*, then as directed above. After cooling, separate carefully the supernatant alcoholic layer from the oil beneath, avoiding concentration, filter, and if necessary restore the original volume of 6 ml. by addition of *absolute ethanol*. Shake the oil layer with 3 ml. of *acetone*, filter the acetone extract on the paper previously used into a measuring cylinder, and wash with three portions of 1 ml. each of *acetone*, adjusting the combined filtrate and washings to exactly 6 ml.

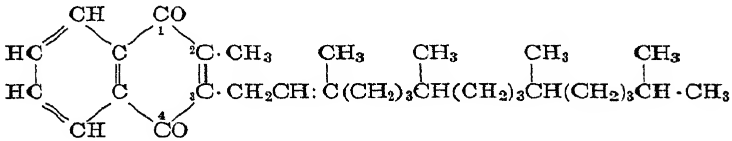
Color Reading and Calculation. Determine separately the color values of the absolute ethanol and the acetone solutions as above. Derive the corresponding amounts of tocopherol from lines plotted on the same standard graph for solutions in the two solvents. The sum of the two readings represents the tocopherol.

Note. Villela [243] substitutes *isobutanol* for ethanol. Pyrocatechol also gives a red color with nitric acid, but this may be removed from the isobutanol by treatment with aluminum hydroxide prepared by the Brockmann method.

Vitamin K

No generally accepted common name has been given the anti-hemorhagic vitamins (K_1 and K_2) present in hog liver, fish muscle, alfalfa, cruciferous leaf vegetables, and various other vegetable foods. According to Fieser,[244] vitamin K_1 is 2-methyl-3-phytyl-1,4-naphthoquinone, or a methyl homolog.

The formula shows relationship to the tocopherols:



Vitamin K_1 (Fieser)[244]

Vitamin K_1 is a light yellow oil that crystallizes from an ethanol solution.

Fieser's [244] formula for vitamin K_2 differs from that for K_1 in that the chain and the CH_3 at 2 are each replaced by the following chain: $CH_2CH{:}[C(CH_3)(CH_2)_2CH]_2{:}C(CH_3)_2$.

Vitamin K is a yellow oil which crystallizes on cooling an acetone solution.

Volumetric Methods. **Trenner and Bacher Indophenol Reduction–Oxidation Volumetric Method.**[245] The method, as devised in the Merck Institute and the Merck Research Laboratories, is in two stages: (1) catalytic reduction of the quinone to hydroquinone in the lower chamber of a special apparatus and (2) reoxidation in the upper chamber of an aliquot with 2,6-dichlorophenol indophenol.

Apparatus. *Trenner and Bacher Apparatus* (Fig. 79). The lower and upper chambers to the ground joint have capacities of 30 and 20 ml. respectively. The conical bottom of the former permits the sweeping of the catalyst into suspension by the gas entering from

J. The rubber bulb (*G*) has a capacity of 30 ml. Tube *K* is 5 mm. outside diameter and is constricted to prevent the cotton plug (*L*) from entering too far. It also contains a wire with a hooked end for inserting and removing

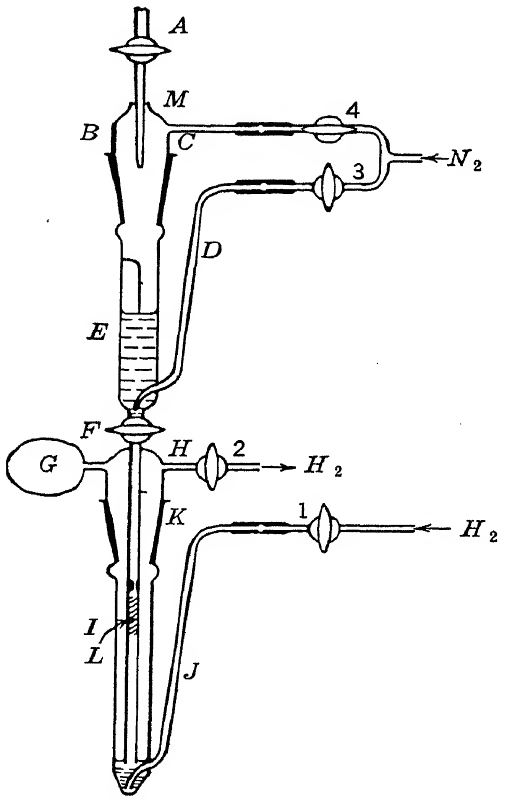


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FIG. 79. Trenner and Bacher Reduction-Oxidation Vitamin K Titration Apparatus.

the plug. A glass pointer in *E* shows a volume of 8 ml. The outlet *M* on head *B* fits the delivery tube of a 5-ml. micro buret.

Electrolytic hydrogen is conducted to *J* through a spiral gas-washing tower containing the same stock solvent (butanol) as used for dissolving the sample. A water seal blow-off adjacent to the tanks prevents excessive pressure.

Included in the nitrogen train is a spiral gas-washing tower containing alkaline sodium hyposulfite solution, prepared from the fresh salt that does not evolve hydrogen sulfide, which removes oxygen that otherwise would reoxidize dihydronaphthoquinone in the aliquot taken for titration.

REAGENTS. *Standard Indophenol Reagent.* Dissolve 1 g. of 2,6-dichlorophenol indophenol (Eastman No. 3463 sodium 2,6-dichlorobenzenone-indophenol) in 1 liter of reagent grade 1.0 *N* butanol by shaking for 15 to 20 minutes, filter, and store in an amber bottle in a refrigerator. Since the dye always contains water and NaCl, the strength of the solution must be determined empirically. The solution keeps 3 months. The dye of certain manufacturers yields a violet-blue solution and is worthless. The stock solution of the dye should be green-blue and when used in the titration no red color should appear at the end-point.

Solvent. For dissolving samples of materials use 95 volume per cent 1.0 *N* butanol containing 0.5 to 1.0 mg. of phenosafranine (Eastman) per liter. The water serves an electrochemical purpose and phenosafranine as a reduction indicator.

Raney Nickel Catalyst. Prepare as described by Adkins and Covert,[246] taking special care to wash the catalyst free from all traces of alkali which destroys both phenosafranine and vitamin K_1. Carry out the final washing with ethanol containing some acetic acid. Remove all "fines" by shaking with a relatively large volume of ethanol, allowing to settle 1 to 2 minutes, and then quickly decanting; repeat until the residual catalyst settles rapidly, leaving no appreciable cloud in the wash liquor. Wash finally on a 400-mesh sieve with a stream of ethanol, rinse into a 15-ml. centrifuge tube, and store under ethanol.

PROCESS. *Charging Apparatus.* Thoroughly clean and rinse with *acetone*. Dry in a vacuum line and insert the plug *L* in *K*. Insert a small cork in *M* and flush out chamber *E* with a current of *nitrogen gas* entering at *C* and leaving at cock *F*. In the meantime support chamber *T* in the stand, start the flow of *hydrogen gas* through *J* (several bubbles per second), then pipet 10 to 12 ml. of the solution of the sample into *I*, add 10 to 15 mg. of *powdered potassium acetate* (serving as a buffer to keep an alkaline reaction), and finally prepared *nickel catalyst*, equivalent to about the bulk of a pea, from which the ethanol was previously removed by centrifuging. The amount of catalyst used should effect complete reduction in 15 to 20 minutes.

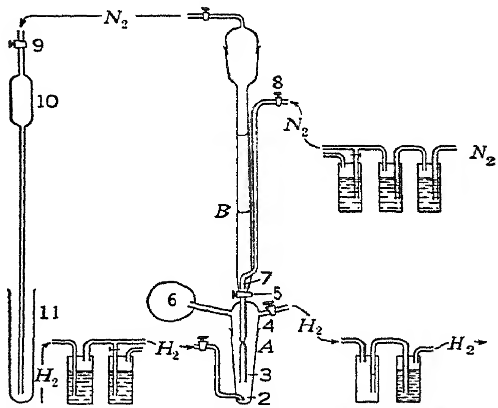
Reduction. With cock 2 open, close *F*, and remove stopper in *M*. Insert buret at *M*, close cock 4, open cock 3, and allow both gas streams to continue. Note that as reduction proceeds the yellowish tinge due to quinone is slowly replaced by the pink of phenosafranine until all the quinone has been reduced, then the pink quickly disappears when the reduction is completed. Continue the reduction 5 minutes after the solution is colorless. During the reduction, squeeze the rubber bulb *G* periodically to expel any air contained therein. Close stopcocks 1 and 2 and allow the catalyst to settle.

Titration. Close cock 3 and open 4, then open *F* and blow the liquid through the cotton plug into chamber *E* by squeezing *G* until the surface of the rising liquid just touches the glass point, as seen by a lens, then close cock *F*, also close 4 and open 3. Allow the *nitrogen gas* to flow during the titration, partly to stir the solution and partly to exclude air. Leucophenosafranine being very sensitive to oxygen gas, the formation of a pink color warns that air has entered. At first add the *indophenol reagent* rapidly, toward the end dropwise. The first drop produces a pink color, the last drop converts the pink-yellow color to greenish blue. The color is stable for not less than 5 minutes. Allow time for the butanol solution to drain before reading.

CALCULATION. Obtain the gammas per milliliter of the vitamin by calculation from the standard previously determined.

***Colorimetric Methods.* Scudi and Buhs Indophenol Oxidation-Reduction Colorimetric Method.**[247] The method (Merck Institute) is a colorimetric adaptation of the principles employed by Trenner and Bacher above, both groups being of the same research organization.

I. Interfering Colors Absent. APPARATUS. *Scudi and Buhs Special Assembly* (Fig. 80).



Courtesy of the Authors and J. Biol. Chem. 1941, **141,** *454*

FIG. 80. Scudi and Buhs Vitamin K Special Assembly.

The lower chamber (*A*) is for the catalytic reduction of 7 ml. of solution, the upper chamber (*B*), outside diameter 12 mm., calibrated at 15 ml., is for the partial reduction of the indophenol reagent by the vitamin K hydroquinone.

Evelyn Colorimeter, with filter 660.

REAGENTS. *Butanol-Acetate Solution*. Dissolve 500 mg. of potassium acetate in 50 ml. of water and dilute to 1 liter with acid-free 1.0 *N* butanol.

Phenosafranine Solution, 0.1%. Prepare a stock solution of 1 mg. of phenosafranine (Eastman No. 1125) per milliliter of water. As needed dilute 1 ml. of the aqueous solution to 100 ml. with 1.0 *N* butanol; 1 ml. = 10 γ.

Raney Nickel Catalyst. See Trenner and Bacher Method above for vitamin K.

Standard 2,6-Dichlorophenol-Indophenol Reagent, 0.05%. Shake for 15 to 20 minutes 50 mg. of the dye (Eastman No. 3463, sodium 2,6-dichlorobenzenone-indophenol) with 100 ml. of butanol and filter through a dry paper with suction. This stock solution, stored in a dark bottle in a refrigerator, keeps 3 months. As needed, dilute about 1 ml. of the stock solution to about 50 ml. with the butanol-acetate solution, and adjust the concentration so that 10 ml. plus 5 ml. of the butanol-acetate solution shows an absorption of 85% in the Evelyn colorimeter with filter 660 on the basis of pure butanol showing 100% transmission.

PROCESS. *Charging Apparatus*. Clean the apparatus with *acetone* and dry on a vacuum line. Place an amount of the *catalyst* half the size of a pea in the bottom of the lower chamber (*A*), add 0.5 to 9.5 ml. of the *butanol-acetate solution* of the sample and *phenosafranine indicator*, and insert cotton in the constricted delivery tube (3). With cocks 8, 5, and 4 open and 1 closed, replace the air in the delivery tube (3) with *nitrogen*, then close cocks 8 and 5 and open 1 and 4. Pass *hydrogen* through a spiral tower containing *butanol* to prevent evaporation losses and insert a water seal blow-off in the line to prevent excessive pressure. Admit *hydrogen* through inlet 2 at such a rate as to keep the catalyst in motion, squeezing bulb 6 from time to time to expel air.

After the quinone is reduced and the phenosafranine has lost its pink color, continue the flow of *hydrogen* for 5 to 10 minutes to insure complete reduction. During the reduction pipet 10 ml. of the *standard indophenol reagent* into the upper chamber and, as the reduction in the lower chamber nearly ceases, remove the air in the indophenol solution with a stream of *nitrogen* freed from oxygen by passing through a spiral tower containing an *alkaline solution of sodium hydrosulfite* ($Na_2S_2O_4$) and *saturated with butanol* contained in another tower. As a precautionary measure, by-pass the gas train with a *butanol* blow-off tower. Admit the *nitrogen* at inlet 7 and allow to bubble through the indophenol solution for 10 minutes, then pass through the long-stemmed pipet (10) placed in the Evelyn colorimeter tube (11) as shown in Fig. 80.

Removal to Upper Chamber. After the reduction is complete, close stopcocks 1, 4, and 8 and by means of rubber bulb 6 force the contents of the lower chamber through the cotton filter in delivery tube 3 into the upper chamber, opening stopcock 5 during the operation and closing it when the solution reaches the 15-ml. mark. Mix the solution by readmitting *nitrogen* through cock 8 for 1 to 2 minutes, then close 8 and 9.

Removal to Colorimeter Tube. By means of a nitrogen-filled pipet (10) connected with a depressed rubber bulb, withdraw the contents of the upper chamber while slowly opening cock 9, then close the cock, remove the rubber bulb, and run the solution through the long stem into the colorimeter tube without splashing.

Color Reading. Take the reading in the colorimeter exactly 3 minutes after the hydroquinone and the indophenol solutions are mixed.

II. Interfering Colors Present (Modified Fieser Method). APPARATUS. *Special Assembly* (Fig. 81), with a capacity of about 100 ml.

REAGENTS. *Claisen's Alkali*. Dissolve 50 g. of KOH in 25 ml. of water and dilute to 100 ml. with methanol.

Other reagents as given under I.

PROCESS. *Reduction*. Dissolve a quantity

of the neutral sample equivalent to 1 to 2 g. of solid matter or oils in a mixture of 5 ml. of *methanol* and 10 ml. of *naphtha*, add 10 to 15 mg. of *powdered phenosafranine*, then transfer the solution to the lower chamber (*C*) of the apparatus and add *Raney nickel catalyst* about equal in bulk to a pea or larger if the reduction is slow. Lead *hydrogen* through a methanol tower to prevent subsequent evaporation losses before it is admitted through the inlet (13) at a rate that keeps the catalyst in motion. A leakage of air into the system is detected by the return of the phenosafranine color.

Formation of the Potassium Salt. After the reduction is complete, place 15 ml. of *Claisen's alkali* in the upper chamber (*D*) and remove air by bubbling *nitrogen* from the inlet 17 through the solution (10 minutes). Admit *Claisen's alkali* through the stopcock (15) and mix the contents of the lower chamber by readmitting the *hydrogen* stream through the stopcock (12), thus forming the potassium salt of the vitamin hydroquinone which remains in the alkaline phase.

Alkali Washing. Cut off the hydrogen supply by closing the stopcock (12), leaving cock 14 open and protecting the system from the entrance of air by connecting the latter with a trap, such as shown in Fig. 80, connected to cock 4. Allow the two phases to separate, then raise the upper chamber through the greased rubber collar (16) so that the naphtha can be removed by connecting an aspirator to the mouth of the upper chamber. Repeat this washing if necessary to complete the removal of neutral fats, vitamin E, and other ether-soluble chromogens.

Hydrolysis and Extraction. Admit 30 ml. of *gas-free water* through the stopcock (15) to hydrolyze the potassium salt of the vitamin K hydroquinone. After mixing for 5 minutes, run in 30 ml. of *gas-free naphtha* through the cock (15), thus extracting the hydroquinone. Mix for 10 minutes with *hydrogen*, then allow the two phases to separate and draw off the lower layer through the stopcock (18). Wash the naphtha layer containing the hydroquinone in the apparatus with 30 ml. of *gas-free water* and remove through cock 18. Run the naphtha phase into a graduated cylinder and measure its

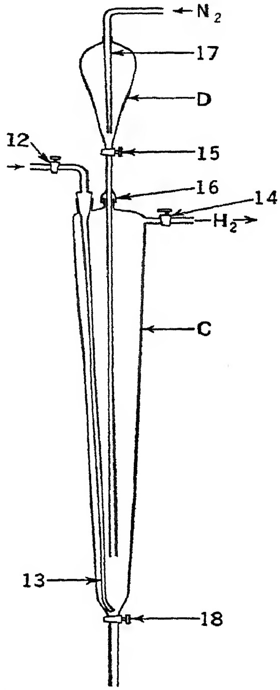


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FIG. 81. Scudi and Buhs Vitamin K Special Apparatus.

volume, then dry the sample over *anhydrous sodium sulfate*. At this point the hydroquinone is exposed for the first time to air oxidation. The small amount of phenosafranine in the naphtha becomes pink but is adsorbed by the sodium sulfate, leaving a practically colorless solution. Finally transfer the vitamin to *butanol* and follow the method as given above under I.

Irreverre and Sullivan Diethyl Dithiocarbamate Colorimetric Method.[248] In a preliminary announcement of work done at Georgetown University, the following details are given.

PROCESS. To 2 ml. of an ethanolic solution of the sample, add 2 ml. of *5% ethanolic sodium diethyl dithiocarbamate solution* and 1 ml. of *ethanolic alkali* (2 g. of sodium in 100 ml. of ethanol). Both vitamin K_1 and 2,3-dimethyl-1,4-naphthoquinone yield a cobalt blue color. The maximum color intensity is reached after 5 minutes and fading begins after 8 minutes.

Polarographic Method. **Herschberg, Wolfe, and Fieser Polarographic Method.**[249] Brief mention is made of the determination of vitamin K_1 dissolved in a mixture of 1 ml. of *isopropanol* and 1 ml. of *0.1 N potassium chloride*, and polarographed at sensitivity *D*.

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11. NATURAL COLORS

(Vegetable and Animal Colors)

The color reactions and specific tests given below for the individual colors or color groups are those which long since were adopted tentatively by the Association of Official Agricultural Chemists. In the *Methods of Analysis* of the Association, the name sodium hyposulfite is assigned to $Na_2S_2O_4$, whereas in *The Merck Index* that formula is given as belonging to the hydrosulfite, the hyposulfite being listed as $Na_2S_2O_3$.

Anthocyanins

The natural colors of red or purple fruits and some vegetables consist largely of anthocyanins which are anthocyanidins (the chromogenic constituents) in combination with a hexose or pentose. With *alkali* the color changes to blue or green, Anthocyanidins, derived by hydrolysis, are largely decolorized by *sodium hydrosulfite*

Flavones

The yellow colors of this group are little changed by *hydrochloric acid, alkali, sodium hydrosulfite* ($Na_2S_2O_4$), *5% uranium acetate solution*, or *sulfuric acid*, but become olive-green or black with *0.5% ferric chloride solution.*

Carotene

Carotene, also xanthophyl and some other carotenoids, are little changed by *hydrochloric acid*, *alkali*, and *sodium hydrosulfite* ($Na_2S_2O_4$), but give a blue color under some conditions.

Annatto

Best known as a vegetable butter color, annatto consists of the pulp of an acid fruit grown in South America and the West Indies. It remains orange on treatment with *hydrochloric acid*, *sodium hydrosulfite*, and *0.5% ferric chloride*, but changes to blue with *sulfuric acid.*

Caramel

Amthor Paraldehyde Test.[1] The test is based on the precipitation with *paraldehyde* and *absolute ethanol*.

Woodman and Newhall Modification.[2] With slight deviation this has been tentatively adopted by the A.O.A.C.

Reagent. *Phenylhydrazine Reagent.* Prepare a solution consisting of phenylhydrazine hydrochloride 2 parts, sodium acetate 3 parts, and water 20 parts.

Process. Add to 10 to 20 ml. of the neutral solution of the color 2 ml. of *5% zinc chloride solution* and 2 ml. of *2% potassium hydroxide solution*, stir well, and centrifuge. Decant the supernatant liquid and add to the precipitate 25 ml. of boiling water. Mix, centrifuge, and again decant, repeating until the wash liquid is colorless.

Dissolve the precipitate in 15 ml. of *10% acetic acid*, concentrate, neutralize carefully, filter, and divide the filtrate into two portions. To one, contained in a 50-ml. glass-stoppered cylinder, add 3 to 5 volumes of *paraldehyde* and just sufficient *absolute ethanol* to form a homogenous liquid. Caramel is indicated by the brown precipitate formed on standing.

To the other portion, add an equal volume of *phenylhydrazine reagent*. A dark brown precipitate is formed if caramel is present.

Turmeric

Boric Acid Test. Slightly acidulate the aqueous or dilute ethanolic solution with *hydrochloric acid* and divide into equal portions. To one portion add *solid boric acid.* A brown-red coloration forms in the presence of turmeric. Compare the color with that of paper treated in like manner without the addition of boric acid. Dip in another portion of the solution a strip of *turmeric paper* and dry on a watch-glass over a water bath. The same brown-red color soon appears. On addition of a drop of *ammonium hydroxide* the color changes to blue-black. This technique is conveniently employed for milk, oysters, sirups, and the water extract of other food products.

Orchil and Cudbear

These lichen dyes are closely related to litmus and give much the same color reactions with acid and alkali.

Tests. Unsulfonated orchil is readily extracted by *amyl alcohol* from a weak acid solution, but the sulfonated dye is incompletely soluble even in a strongly acid solution. The color changes with *acid* and *alkalies* are similar to those for cochineal except that *sodium hydrosulfite* ($Na_2S_2O_4$) decolorizes orchil, but the original color is restored by air oxidation. Dyeing tests employing stripping of the color may also be applied.

Chlorophyl

Petering, Wolman, and Hibbard Acetone–Barium Hydroxide Photometric Method. Process. *Acetone Extraction.* See method for carotene and chlorophyl by the same authors above (C10).

Reading. Compare the transmission of a portion of the acetone extract with that of the solvent or water in a 1.0-cm. absorption cell of the instrument, using a Corning No. 243 H.R. signal red filter, 4.35 mm. thick, and a No. 396 H.R. Aklo polished glass filter, light shade, 2.50 mm. thick. This combination permits the isolation from a tungsten lamp of radiation above 6100 Å only, removes much of the infra-red from the lamp, and transmits radiation in the region where chlorophyl has the maximum absorption, thus permitting precise measurement of the chlorophyl concentration.

Calculation. Convert the transmission into concentration by means of a standard calibration curve obtained with pure chlorophyl in a concentration below 60 γ/ml.

Comar and Zscheile Spectrophotometric Method.[3] Advantage is taken of the difference in the relative specific absorption coefficients in ether solution of the components at 6600 and 6425 Å, which are respectively 102 and 16.3 for *a* and 4.50 and 57.5 for *b* chlorophyl.

Apparatus. *Waring Blendor.*

Scrubbing-Tubes. Open tubes about 20 mm. in diameter, to each of which is sealed a narrower tube drawn down to a jet at the end.

Cenco-Sheard Spectrophotelometer, or equivalent instrument capable of isolating a spectral region of about 30Å near 6600 Å with negligible stray radiation.

Reagents. *Commercial Ether,* purified for fat extraction.

Trisodium Phosphate. Wash all glass ware with the concentrated solution.

Process. *Maceration and Acetone Extraction.* Disintegrate 2 to 10 g. of the fresh sample mixed with about 0.1 g. of *calcium carbonate* in a Waring Blendor or by grinding with sand in a mortar. Add *85% acetone* and continue the maceration. Filter through quantitative paper in a Büchner funnel and wash with *85% acetone* also, if necessary, a little *ether*. Repeat the treatment until the extraction is complete.

Transfer to Ether. Pour the combined extracts into a volumetric flask of suitable size, dilute to the mark with *85% acetone*, and pipet 25 to 50 ml. into a separatory funnel containing 50 ml. of *ether*. Carefully add water until all the fat-soluble pigments are transferred to the ether layer, then draw off and discard the aqueous layer. Deliver the ether solution dropwise through a scrubbing-tube to the bottom of a second separatory funnel containing about 100 ml. of water, rinsing with *ether* added from a medicine dropper. Transfer the scrubbing-tube to the first funnel and draw off and discard the water in the second funnel, then deliver the ether solution as before through the scrubbing-tube into the first funnel containing about 100 ml. of water. Continue this washing five to ten times until all the acetone is removed from the ether solution. Transfer the ether solution to a 100-ml. volumetric flask, dilute to the mark with *ether*, and mix.

Spectrophotometric Measurement. Fill a dry 60-ml. reagent bottle, containing a teaspoonful of *anhydrous sodium sulfate*, with the ether solution. When clear, remove an aliquot to a second dry bottle and dilute with sufficient dry *ether* to allow the $\log_{10} (I_0/I)$ value to fall between 0.1 and 0.8 (preferably 0.6) at wave length 6600 Å.

Select two glass-stoppered tubulated absorption cells that give the same galvanometer deflection when filled with dry ether, after cleaning the outside surface with wet and then with dry cotton. Empty one, fill with the ether solution, and examine in the instrument with entrance and exit slits adjusted to 30 to 40 Å at 6600 Å. Check the adjustment by taking readings through the solution and solvent at intervals of 10 Å from 6580 to 6650 Å. For grating instruments, apply the same correction at 6425 Å, but for prism instruments obtain a special correction. Take I_0 and I readings of the unknown at 6600 and 6425 Å or the corrected setti

CALCULATION. Substitute in the following equations the $\log_{10} (I_0/I)$ values calculated for the readings:

$$\text{(1) Total chlorophyl } (\gamma/\text{ml.}) = 7.12 \log_{10} \frac{I_0}{I} \text{ (at 6600 Å)} + 16.8 \log_{10} \frac{I_0}{I} \text{ (at 6425 Å)}$$

$$\text{(2) Chlorophyl } a\ (\gamma/\text{ml.}) = 9.93 \log_{10} \frac{I_0}{I} \text{ (at 6600 Å)} - 0.777 \log_{10} \frac{I_0}{I} \text{ (at 6425 Å)}$$

$$\text{(3) Chlorophyl } b\ (\gamma/\text{ml.}) = 17.6 \log_{10} \frac{I_0}{I} \text{ (at 6425 Å)} - 2.81 \log_{10} \frac{I_0}{I} \text{ (at 6600 Å)}$$

EXAMPLES. Comar reports as follows: total chlorophyl, alfalfa 2.45 and string beans 1.42 mg./g., and chlorophyl *a* in the total chlorophyl, alfalfa 71.4 and string bean 73.6%.

COCHINEAL

Although consisting of dried insects and only indirectly derived from plants, cochineal is conveniently grouped with vegetable colors.

Robin Uranium Acetate Color Test. Acidify the sample with one-third its volume of *hydrochloric acid*, shake with *amyl alcohol*, and separate the solvent layers. Remove the acid from the amyl alcohol solution by washing several times with water and dilute with 1 to 2 volumes of *naphtha*. Shake the solvent mixture with several small portions of water and divide the aqueous solution of the dyes thus obtained into two portions.

To one portion add a little *sodium acetate*, then dropwise *5% uranium acetate solution*, shaking well after each addition. If cochineal is present, a characteristic emerald green coloration is produced.

[illegible] nation to the other portion add

1 or 2 drops of *ammonium hydroxide;* this causes the well-known change in color due to cochineal. Many fruit colors, however, show a similar change in color.

Sodium Hydrosulfite Test. Cochineal is distinguished from orchil in that it is not decolorized with $Na_2S_2O_4$ in an acid, alkaline, or neutral solution.

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[3] *Plant Physiol.* 1941, **16,** 651; 1942, **17,** 198; Comar: *Ind. Eng. Chem., Anal. Ed.* 1942, **14,** 877; *J. Assoc. Official Agr. Chem.* 1944, **27,** 78; Tentative A.O.A.C. Method.

12. ARTIFICIAL COLORS

Pros and Cons of Artificial Colors. Dr. Wiley once remarked to one of us that the food dyer had less of an excuse for his craft than the food preserver, referring in the latter case to the use of chemical preservatives. Dyes serve no useful purpose other than esthetic or distinctive, whereas preservatives, however objectionable from the hygienic viewpoint, at least permit long keeping and shipment to the ends of the earth with retention of palatability and without loss of nutrients. In the days of unrestricted adulteration colors served to cover many a gross fraud and today, even when declared, they put the natural uncolored food at a disadvantage. For distinguishing candies and other articles of different flavors, however, a variety of harmless colors seems useful and quite proper.

Legal Control. Before the enactment of the Federal Pure Food Law the widespread coloring of foods with dyes was neither prohibited nor legalized and little attention was paid to analytical methods in the United States. In the early regulations for the enforcement of the law, harmless vegetable dyes, one animal (cochineal), and eight coal-tar colors were allowed in food provided the latter were certified and their presence in the food was declared. This rule necessitated the analysis of the colors with reference to their identity and freedom from foreign matter injurious to health and the equally exacting analytical examination of foods for the presence and kind of color.

Analytical Schemes and Identification Tables. Witt,[1] Weingartner,[2] and Rota [3] prepared analytical schemes widely used in Europe. Winton [4] applied these schemes to products on the American market as early as 1900. Mathewson [5] gives a scholarly scheme for the separation and identification of dyes used in the United States, including the eight original permitted colors, which in condensed form was inserted in the fourth edition of Leach and Winton *Food Inspection and Analysis.* Loomis, Mathewson, and others have prepared tables for identification of the dyes after fixing on wool. This vast amount of highly technical work has lost much of its significance now that the number of permitted colors has been increased to seventeen.

To meet the changed conditions, a new scheme and table, developed in the laboratory of the U. S. Food Administration, has been tentatively adopted by the A.O.A.C. in the form given below.

Since the presence in foods of dyes, other than those legally permitted, is now extremely rare, the extensive early literature on their detection seems of little value. The subject matter which follows is accordingly limited largely to (1) tests for such few natural dyes as are most commonly used where, because of prejudice or law, coal-tar dyes are in disfavor, (2) the analytical scheme for the separation of the permitted colors, and (3) the two-page table for identification of the colors on the fibers, all substantially as tentatively adopted by the Association.

Coal-Tar Colors

Separation by Dyeing. **Arata Bisulfate Wool Dyeing Test.**[6] One of us used this test to illustrate the extent to which coal-tar colors were, and doubtless still are, used in food products. From single glasses of soda water flavored with imitation sirup enough of

the dye was fixed on a piece of nun's veiling the size of a pocket handkerchief to impart brilliant hues of red, magenta, violet, green, orange, and yellow; from jams, jellies, and cordials similar results were obtained.

PROCESS. Dilute 25 to 50 ml. of the material to 100 ml., boil for 10 minutes with 10 ml. of *10% potassium bisulfate solution* and a piece of *white nun's veiling* or *white woolen yarn* previously sensitized by boiling with *1% sodium hydroxide solution* and washing. Wash the dyed cloth or yarn in boiling water and dry between folds of filter paper.

If the coloring matter is natural, the fibers will take on a faint pink or brown color, changing to green with *ammonium hydroxide* and not restored by washing; basic coal-tar colors such as acid magenta, the tropeolins, ponceaus, and numerous other azo colors are fixed on the wool and the color is not changed by ammonia or, if changed, is restored by washing.

Make spot tests on the pieces either directly or, better, after a second dyeing as in the following method.

Sostegni and Carpentieri Hydrochloric Acid Dyeing Test.[7] PROCESS. To 100 ml. of the solution obtained as by the Arata method, add 2 to 4 ml. of *10% hydrochloric acid* and boil for 5 to 10 minutes with a piece of *nun's veiling* or *white woolen yarn*. Wash the cloth or yarn with hot water, then boil with *very dilute hydrochloric acid* and again wash. Extract the color from the fibers by boiling in *1 + 50 ammonium hydroxide*, remove the old cloth or yarn, and repeat the dyeing with fresh pieces. By this second dyeing all vegetable colors are eliminated and spot tests of the coal-tar colors may be made on the dyed fibers in accordance with the table.

A.O.A.C. Modification. The test has been extended to include basic as well as acid dyes and it has been adopted for various food products.

PROCESS. (*a*) *Wines, Fruit Juices, Distilled and Malt Liquors, Flavoring Extracts, Vinegar, Non-Alcoholic Beverages, and Similar Products.* Dilute 20 to 200 ml. of the sample with 1 to 3 volumes of water, neutralize with *1 + 9 ammonium hydroxide* if necessary, add a piece of *sensitized nun's veiling* and boil (or heat on a steam bath) until the ethanol is largely removed or, if no ethanol is present, for 5 to 15 minutes. Remove the cloth and rinse with water. To the solution add 3 or 4 drops of *hydrochloric acid* for each 100 ml., introduce a new piece of *cloth*, and repeat the boiling.

Basic colors dye best from neutral or faintly ammoniacal solutions, acid dyes from neutral or acid solution. Lichen colors (orchil, cudbear, and litmus), turmeric, and some other vegetable colors dye wool to some extent; on the other hand, some coal-tar dyes, such as auramine and naphthol green B, may not impart a strong color.

Remove the dye from the cloth by boiling with a little *1 + 9 ammonium hydroxide*, squeeze out the adhering liquid, and boil off the ammonium hydroxide from the solution, then introduce a new piece of *cloth*, acidify with *1 + 9 hydrochloric acid*, and repeat the dyeing once or if necessary a second time.

(*b*) *Other Food Products.* Treat fruit products after dilution as above. Separate the colored parts of candies from the uncolored. Extract canned and preserved fruit and vegetables, sausage casings, smoked fish, coffee, etc., with *70% ethanol*, containing *1% sodium hydroxide solution*, and examine the extract. Alternately dye and strip cocoa products from *70% ethanol* several times to remove natural colors.

A.O.A.C. Methods of Separation by Immiscible Solvents and Other Reagents. Two schemes, one short covering permitted dyes and others, the other more in detail but restricted to the permitted colors, have been tentatively adopted.

Short Scheme. A. Basic Coal-Tar Dyes. Most basic dyes may be separated from mixtures by making alkaline with *10% sodium*

hydroxide solution and shaking with *ether*. Separate the ether layer, which may or may not be colored, wash twice with a few milliliters of water to remove excess alkali, and shake with *1 + 18% acetic acid*, thus obtaining a colored solution. Although perhaps altering the color, the treatment serves to detect methyl violet B (451), magenta (448), Bismarck brown (197), malachite green (427), and rhodamine B (504), also auramine (425), although the latter quickly decomposes in alkaline solution.

B. Acid Coal-Tar Dyes. If dyes other than or in addition to those permitted are suspected, their partial separation may be effected by shaking an amyl alcohol extract of the aqueous solution with half the volume of *hydrochloric acid*, followed by shaking successively with half the volume of water, thereby fractioning the dyes according to their solubility in different proportions of the acid. Ponceau 6R (108) is soluble at about normal acidity; amaranth (107), brilliant scarlet (106), tartrazine (94), sunset yellow FCF, orange G (14), and soluble blue (480) are soluble at about 0.25 *N*; and palatine scarlet (53), ponceau 2R (55), ponceau 3R (56), ponceau SX, naphthol yellow S (4), cochineal (706), crystal ponceau (64), and azorubine A (103) are soluble at 1/16 to 1/256 *N*.

When practically all the acid is removed, orange I and II (85 and 86) and crocine orange (13) begin to wash out, and less readily orange IV (88) and metanil yellow (95). Unsulfonated dyes such as erythrosin G and B (516 and 517) and rose bengal (520 and 523) are removed very slowly by water, if at all, unless the solvent is diluted with *naphtha* and the dyes are removed with water containing a few drops of *ammonium hydroxide*. Acid yellow (8) and brilliant yellow S (89), also indigotin (692) are partially taken up by *amyl alcohol* from an acid solution and appear chiefly in the first washings.

Detailed Scheme for Separation of Permitted Colors. REAGENTS. *Various Solvents:* ether, ethanol, amyl alcohol, butanol, naphtha ("low b.p. gasoline"), carbon tetrachloride, α-dichlorhydrin, ethyl acetate, and amyl acetate.

Formaldehyde-Acetic Anhydride Reagent: 1 part 40% formaldehyde + 4 parts acetic anhydride.

Acid Sulfate Reagent. Dissolve 100 g. of anhydrous Na_2SO_4 and 13.5 ml. of H_2SO_4 in water and dilute to 1 liter.

A. Separation of Oil-Soluble Permitted Colors. Shake the sample (usually an oil or fat) with an equal volume of *90% (by volume) ethanol* and wash the extract with several portions of *naphtha*. Evaporate to dryness the ethanol extract (containing aniline yellow, butter yellow, aminoazotoluene, auramine, sudan, yellow OB, yellow AB, etc.), take up with 40 ml. of *naphtha*, and shake with two or three portions of 5 ml. each of *2 to 4% sodium hydroxide solution* (to remove any annatto, turmeric, etc.).

Extract the naphtha solution (containing any yellow OB, yellow AB, orange SS, and oil red XO) three times with half its volume of *13 N sulfuric acid* and shake each acid extract with two portions of equal volumes of *naphtha*, using the same two portions for the three acid extracts. Then extract each of the latter naphtha portions with 20 ml. of *13 N sulfuric acid*, using the same portions for each. Extract the second of these naphtha portions with another 20-ml. portion of *13 N sulfuric acid*. Combine the three acid extracts as obtained by shaking the three naphtha solutions three, four, and five times respectively, wash with water, reextract with *naphtha*, and evaporate the solvent, thus obtaining the pure yellow AB. Combine the original naphtha solution and the acid-washed naphtha solutions, wash with small portions of water to remove the excess of acid, and evaporate the solvent, thus obtaining yellow OB. Shake a 5-ml. neutral

naphtha solution of each of the nearly pure dyes with 5 ml. of *formaldehyde-acetic anhydride reagent.* In a few seconds yellow AB gives a red solution and yellow OB an orange solution.

B. Separation of Other Permitted Colors. Obtain a solution of the dyes (preferably 0.01 to 0.05%) as directed for the dyeing test, but omitting the fixation of the color on the wool, avoiding as far as possible contamination with suspended matter, ethanol, acids, alkalies, and salts.

Add *25% sodium chloride solution* to about 10% concentration and *acetic acid* to 1/4 of its volume. Extract with 3- to 50-ml. portions of *amyl alcohol*, draw off the lower layer, and reserve for further treatment. Wash the amyl alcohol extract with 25-ml. portions of *5% sodium chloride solution* until the washings are colorless or nearly so and add the washings to the original aqueous solution. Dilute the amyl alcohol extract with an equal volume of *naphtha* and wash with 25-ml. portions of water until all color (orange I, guinea green B) is extracted and separate as directed below (1). Treat the amyl alcohol-naphtha solution with 10-ml. portions of *0.1 N sodium hydroxide solution* (or *1 + 9 ammonium hydroxide*) to remove erythrosin. Acidify the original solution and washings (from which the three dyes were removed) with 1/40 volume of *hydrochloric acid* and extract in 50-ml. volumes with three 50-ml. portions of *amyl alcohol*. Reserve the lower aqueous layer for further treatment.

Wash the amyl alcohol extract with 25-ml. portions of *0.25 N hydrochloric acid* until the washings are nearly or quite colorless and add the washings to the aqueous extract. Extract the amyl alcohol with several 25-ml. portions of water until all color is extracted. For the separation from the solution of ponceau 3R, ponceau SX, and naphthol yellow S, see (2) below.

Treat the original solution and washings (from which the above 6 dyes were removed) in 50-ml. volumes with 3- to 50-ml. portions of *α-dichlorhydrin*. Reserve the upper aqueous layer for further treatment. Wash the dichlorhydrin extract with several 20-ml. portions of *25% sodium chloride solution* and combine the washings with the aqueous solution above. Dilute the dichlorhydrin extract with 2 volumes of *carbon tetrachloride* and extract with several 25-ml. portions of water until all color (light green SF yellowish, fast green FCF, and brilliant blue FCF) is extracted. See (3) below for their separation.

Further acidify the original solution and washings from which the above 9 dyes were removed with 1/40 its volume of *hydrochloric acid* and extract in 50-ml. volumes with three 50-ml. portions of *amyl alcohol*. Reject the lower colorless, or nearly so, layer and wash out the dyes from the amyl alcohol extract with several 25-ml. portions of water until all color (indigotin, amaranth, tartrazine, and sunset yellow FCF) is removed. For their separation see (4) below.

(1) Orange I and Guinea Green B. Extract the combined colors with two 20-ml. portions of *α-dichlorhydrin* and reject the colorless upper aqueous layer. Dilute the solvent layer with 2 volumes of *carbon tetrachloride* and extract orange I with several 10-ml. portions of water and guinea green B with several 10-ml. portions of *25% ethanol.*

(2) Ponceau 3R, Ponceau SX, and Naphthol Yellow S. Acidify the solution of the combined colors with 1/10 its volume of *hydrochloric acid* and extract the naphthol yellow S with two 20-ml. portions of washed *ethyl acetate* or *amyl acetate.* Ponceau 3R and ponceau SX remain almost entirely in the aqueous layer. Wash the solvents with 5-ml. portions of *1.0 N hydrochloric acid* to remove traces of the ponceaus. Remove naphthol yellow S from the combined ethyl acetate or amyl acetate extract with 5-ml. portions of *1 + 9 ammonium hydroxide.* Extract the remaining ponceau solution with

20-ml. portions of *amyl alcohol* and wash out the excess of acid with a few small portions of water. Dilute the amyl alcohol with an equal volume of *naphtha* and remove the color with small volumes of water. Treat 10 ml. of the solution successively with 1 ml. of *hydrochloric acid*, 2 ml. of *strong bromine water*, and 3 ml. of *saturated hydrazine sulfate solution*, then pour immediately into a test tube containing 10 ml. of *2 N sodium carbonate solution* and 2 drops of *1% ethanolic α-naphthol;* a light orange-colored solution indicates ponceau 3R; a deep brown-red solution indicates ponceau SX.

Add to the solution 5 ml. of *ether*, mix well, and draw off the lower aqueous layer containing, if colored, ponceau SX. Add to the ethereal extract an equal volume of *hydrochloric acid;* the formation of a purplish solution confirms the presence of ponceau 3R.

(3) Light Green SF Yellowish, Fast Green FCF, and Brilliant Blue FCF. Treat the combined colors with an equal volume of *2 N sodium carbonate solution* and extract in 25-ml. volumes with two 50-ml. portions of *1.0 N butanol.* Draw off the lower aqueous layer containing the fast green FCF and wash out the last traces from the solvent with 25-ml. portions of *2 N sodium carbonate solution.* Combine the washings and the aqueous solution for confirmatory test. Light green SF yellowish is colorless in the solvent, whereas brilliant blue FCF imparts a bluish green. As proof of the presence of light green SF yellowish in the presence of brilliant blue FCF, proceed as follows. Dilute with an equal volume of *naphtha* and remove the color with small portions of water. Boil for 5 minutes 20 ml. of the solution with 4 ml. of *10% sodium hydroxide solution.* Brilliant blue FCF changes to red, whereas light green SF yellowish changes to yellow. Acidify with 10 ml. of *glacial acetic acid;* brilliant blue FCF changes to violet and light green SF yellowish to green. Heat with 3 g. of *zinc dust* until the solution is colorless, filter, and make slightly alkaline with *ammonium hydroxide*, then acidify with *acetic acid* and bring to a boil; light green SF yellowish is indicated by a deep green color, whereas brilliant blue FCF remains colorless.

(4) Indigotin, Amaranth, Tartrazine, and Sunset Yellow FCF. Heat to boiling a small portion of the neutral or faintly acid solution and add a few crystals of *sodium hydrosulfite* until all the dye is reduced, then add a few drops of *glacial acetic acid* and shake with air; the indigotin is quickly restored, whereas amaranth, tartrazine, and sunset yellow FCF are destroyed. If a positive test for indigotin is obtained, add to the remainder of the mixed dye solution several decigrams of *urea*, heat, and while boiling add 1 or 2 drops of *10% sodium nitrite solution;* indigotin is converted to pale yellow isatin sulfonate, whereas amaranth, tartrazine, and sunset yellow FCF are but little affected. Acidify the resultant mixture with 1/10 its volume of *1 + 4 sulfuric acid*, extract in 25-ml. portions with three 50-ml. portions of *1.0 N butanol*, draw off the lower layer, and pour successively through all the funnels. Reserve the aqueous layer, if colored; otherwise reject. Extract the butanol solution with 25-ml. portions of the *acid sulfate reagent* until the washings are colorless and reserve the washings for the separation of amaranth and tartrazine. Dilute the butanol solution with an equal volume of *naphtha* and remove the sunset yellow FCF with water. Confirm with dyeing tests and wet reactions.

Acidify the reserved solution with 1/20 its volume of *hydrochloric acid* and shake with two 30-ml. portions of *amyl alcohol;* amaranth and tartrazine are extracted. Reject the lower layer containing the isatin compound. Remove the coloring matter with several 10-ml. portions of water. To one portion, add 5 drops of *ammonium hydroxide* and a few

Color Reactions on Dyed Fibers by Various Reagents (A.O.A.C.)

(Permitted colors are in bold-faced type.)

Coloring Matter	C. I. No.	S. & J. No.	Strong Hydrochloric Acid	Concentrated Sulfuric Acid	10% Sodium Hydroxide Solution	Dilute Ammonium Hydroxide
Rhodamine B	749	504	Orange	Yellow	Bluer	Bluer
Rose Bengal	779	523	Almost decolorized	Orange	No change	No change
Archil	1242	710	Red	Reddish brown	Violet	Violet
Magenta	677	448	Yellowish brown	Yellowish brown	Decolorized	Paler
Acid magenta	692	462	Almost decolorized	Yellow	Decolorized	Decolorized
Palatine red	85	62	Darker	Blue	Dull brown	Little change
Bordeaux B	88	65	Violet	Blue	Brick red	Little change
Amaranth	184	107	Slightly darker	Violet to brownish	Brown to orange-red	Little change
Azorubine A	179	103	Little change	Violet	Red	Red
Erythrosin	773	517	Orange-yellow	Orange-yellow	No change	No change
Ponceau 6RB	286	169	Blue	Blue	Dull violet-red	Little change
Ponceau 6R	186	108	Violet-red	Violet	Brown	Orange-red
Crystal ponceau	89	64	Red	Violet	Dull brown	Little change
Ponceau 3R	80	56	Little change	Little change	Dull orange	Little change
Ponceau SX	...	...	Deeper red	Deeper red	Orange yellow	Orange yellow
Sudan III *	248	143	Violet, then brown	Green	Violet-red	Little change
Safranine	841	584	Greenish blue	Green	Red	Red
Brilliant scarlet	185	106	Red	Violet-red	Yellowish brown	Orange-red
Ponceau 2R	79	55	Little change	Little change	Brownish yellow	No change
Palatine scarlet	77	53	Darker	Violet-red	Brownish yellow	No change
Erythrosin G	772	516	Yellow-orange	Yellow-orange	No change	No change
Sudan II *	73	49	Red	Violet-red	Little change	No change
Sudan I *	24	11	Orange-red	Red	Redder	No change
Cochineal	1239	706	Little change	Little change	Violet-red	Violet-red
Bismarck brown	331	197	Redder, darker	Browner	Yellower	Yellower
Bismarck brown R	332	201	Redder, darker	Browner	Yellower	Yellower
Orange I	150	85	Violet	Violet	Red, dark	Red, dark
Orange II	151	86	Red	Red	Dull red	No change

Croceine orange	*26*	13	Orange-red	Orange	Slightly darker	No change
Orange G	*27*	14	Little change	Orange	Dull, brownish red	No change
Yellow OB * †	*61*	...	Red	Violet	Little change	No change
Yellow AB * ‡	*22*	...	Red	Violet	Little change	No change
Sudan G *	*23*	10	Orange-yellow	Brownish yellow	Orange-yellow	No change
Butter Yellow *	*19*	16	Violet-red	Orange-yellow	No change	No change
Aniline Yellow *	*15*	7	Violet-red	Orange-yellow	Little change	No change
Aminoazo-*o*-toluene *	*17*	...	Dull orange	Orange-yellow	Little change	No change
Fluorescein	*766*	510	Little change	Little change	Green fluorescent	Green fluorescent
Metanil yellow	*138*	95	Violet-red	Violet	No change	No change
Azoflavine	*145*	92	Violet-red	Violet-red	Dull brown	Little change
Acid yellow	*16*	8	Red	Orange	Little change	No change
Brilliant yellow S	*144*	89	Violet-red	Violet-red	Little change	Little change
Tartrazine	*640*	94	Slightly darker	Slightly darker	Little change	Little change
Sunset yellow FCF	...	...	Slightly redder	Slightly redder	Browner	No change
Naphthol Yellow S	*10*	4	Almost decolorized	Pale brown	No change	No change
Auramine	*655*	425	Decolorized	Almost decolorized	Decolorized	Paler
Turmeric	*1238*	707	Red	Reddish brown	Orange	Orange
Quinoline yellow	*801*	667	Slightly darker	Brownish yellow	Slightly paler	Little change
Naphthol green B	*5*	398	Yellowish	Brownish yellow	No change	No change
Guinea green B	*666*	433	Pale orange-yellow	Yellowish brown	Decolorized	Decolorized
Light green SF yellowish	*670*	435	Pale orange-yellow	Yellowish brown	Decolorized	Decolorized
Fast green FCF	...	...	Orange	Green to brown	Blue	Blue
Brilliant blue FCF	...	...	Yellow	Yellow	No change	No change
Night green 2B	*667*	438	Pale orange-yellow	Yellowish brown	Decolorized	Paler
Malachite green	*657*	427	Almost decolorized	Almost decolorized	Decolorized	Decolorized
Erioglaucine A	*671*	436	Yellow	Pale, dull yellow or brown	Slightly darker	Little change
Patent blue A	*712*	442	Pale orange-yellow	Green to brown	Little change	Little change
Soluble blue	*707*	480	Paler	Brown	Pale reddish	Almost decolorized
Indigotin	*1180*	692	Slightly darker	Darker	Greenish yellow	Greenish blue
Formyl violet	*698*	468	Pale orange-yellow	Pale, dull orange	Decolorized	Decolorized
Methyl violet	*680*	451	Yellowish	Yellowish	Decolorized	Almost decolorized
Nigrosine, soluble	*865*	602	Dull bluish	Dull greenish	Brownish red, paler	Pale reddish

* Oil-soluble. † *o*-Tolueneazo-β-naphthylamine. ‡ Benzeneazo-β-naphthylamine.

crystals of *sodium hydrosulfite;* this destroys the amaranth, leaving the tartrazine practically unaltered. Acidify with *hydrochloric acid* and extract speedily with a small volume of *amyl alcohol* from which tartrazine can be separated with *0.25 N hydrochloric acid.* Treat another 10-ml. portion of the neutral dye solution in a test tube with 2 ml. of *20% ammonium chloride solution* and 1 ml. of *25% potassium cyanide solution,* then heat in a boiling water bath for 5 minutes. Cool rapidly, acidify with 2 ml. of *hydrochloric acid,* and extract cautiously with 10 ml. of *amyl alcohol.* Draw off the lower layer and reject. Remove tartrazine with 5-ml. portions of *0.25 N hydrochloric acid;* amaranth is converted to a lower sulfonated dye not removable at that concentration. Dilute the solvent with an equal volume of *naphtha* and extract the dye with small volumes of water; amaranth is modified to a brownish red dye.

Identification of Colors by Reagents Applied to the Dyed Fibers. Transfer the dye to small pieces of *woolen cloth* (nun's veiling) or, in the case of oil-soluble colors, to *silk,* by boiling the solution (20 to 200 ml.) after adding *1 + 9 ammonium hydroxide* to neutral or faintly alkaline reaction (basic dyes) and again after treating with 3 or 4 drops of *hydrochloric acid,* as in the Sostegni and Carpentieri test, or with 10 ml. of *10% potassium bisulfate solution,* as in the Arata test. Wash the dyed cloth thoroughly in running water, dry, cut in pieces, and apply to separate pieces the following reagents: *hydrochloric acid, sulfuric acid, 10% sodium hydroxide solution,* and *12% ammonium hydroxide.* Compare the color changes with those given in the A.O.A.C. table above and those produced by the same treatment on cloth dyed with 0.1 to 0.5% solutions of standard colors.

Special Tests for Permitted Colors. See *Methods of Analysis, A.O.A.C.*

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13. CHEMICAL PRESERVATIVES

Benzoic Acid

Ferric Chloride Test. The test has long been an official and longer still a much used method of detection.

Reagent. *Neutral Ferric Chloride Solution.* Add to a 0.5% solution of $FeCl_3 \cdot 6H_2O$ dilute NH_4OH until a slight precipitate forms and filter.

Process. *Extraction.* If alcohol is absent, place 50 ml. or 50 g. in a separatory funnel and make distinctly acid with *hydrochloric acid;* if alcohol is present, weigh or measure the portion into an evaporating dish, add *sodium hydroxide solution* to alkaline reaction, dealcoholize, restore the original volume, make distinctly acid to *litmus* with *hydrochloric acid,* and transfer to the separatory funnel. In all cases shake with an equal volume of *ether,* draw off the aqueous layer, and wash the ether extract with several small portions of water, centrifuging or adding a little *naphtha* to destroy an emulsion if necessary. Evaporate the ether extract at room temperature. Note if crystalline plates of benzoic acid are evident.

Ferric Chloride Treatment. Add to the dry residue *ammonium hydroxide* to alkaline reaction, evaporate the excess on the water bath and take up in a few drops of water, then add a few drops of *neutral 0.5% ferric chloride solution.* The presence of benzoic acid is indicated by the formation of a light red-brown precipitate.

Mohler Nitrate-Sulfide Test.[1] The test, as modified by Heide and Jakob,[2] is as follows. To the dry ether extract obtained as in the ferric chloride test (phenolphthalein must be absent), add 1 to 3 ml. of *0.33 N sodium hydroxide solution* and again evaporate to dryness. Add to the residue 5 to 10 drops of *sulfuric acid* and a small crystal of *potassium nitrate,* then heat for 10 minutes in a glycerol bath at 120 to 130°, thus forming *m*-dinitrobenzoic acid. Cool, add 1 ml. of water, make alkaline with *ammonium hydroxide,* and boil to decompose any ammonium nitrite that may have been formed. Cool, add a drop of fresh colorless *ammonium sulfide* without mixing. A red-brown ring of *m*-diamidobenzoic acid is indicative of benzoic acid. On mixing, the color diffuses and on heating changes to greenish yellow, thus distinguishing benzoic from salicylic or cinnamic acids, both the latter forming amido compounds which are not changed by heating.

Peter Oxidation Test.[3] If no salicylic acid is present, oxidize the benzoic acid to that acid by dissolving the ether extract, obtained as for the ferric chloride test, in a test tube in 5 to 8 ml. of *sulfuric acid* with the addition of 0.5 to 0.8 g. of *barium peroxide* in small portions with shaking under the tap. After 30 minutes, dilute the solution, shake, cool, filter, extract with *ether,* and proceed as directed for salicylic acid below.

Monier-Williams Metallic Magnesium Test.[4] The fixation of benzoic acid vapors by metallic magnesium is applied by Monier-Williams in a test that, if not strictly quantitative, distinguishes large amounts from traces.

Process. The steps are essentially the same as those of the Mix modification up to the side-head Titration, except that (1) a methylated 1 + 1 ether-naphtha mixture is used instead of 1 + 1 ethyl acetate-naphtha mixture for the extraction of the benzoic acid and (2) the extracted benzoic acid is sublimed and weighed as follows:

Sublimation. Avoiding violent shaking, transfer the extract to a stoppered test tube by suction through one of two tubes in a stopper as in the Werner-Schmidt apparatus for fat determination in milk. Wash the delivery tube with the solvent after the extraction, immerse the test tube in water at 30°, and remove the solvent in a current of dry air (1 hour). Mix the residue with 2 g. of washed and ignited sand, place a disk of filter paper in the test tube about 4 cm. above the bottom, loosely stopper, introduce the test tube into an air bath through a hole in the metal cover, and heat from 1 to 1.5 hours at 160°. Cut off the test tube below the filter paper and add to the sublimate in the upper portion any crystals caught in the paper. Dry the upper portion of tube plus sublimate in a desiccator and weigh.

ACCURACY. The error is given as 1 to 2 mg. in 20 to 60 ml. Benzoic acid may be formed from cinnamic acid, but not from saccharin in appreciable amount.

Determine salicylic and cinnamic acids by the same method, omitting the oxidation which destroys the former and converts the latter partly into benzoic acid. Use the Autenrieth and Benttel method [5] and the de Jong method [6] for the determination of salicylic and cinnamic acids respectively in the presence of benzoic acid and each other.

Mix Quantitative Modification.[7] APPARATUS. *Assembly*, consisting of (1) a boiling flask, (2) a bulb reflux condenser, (3) a large test tube, constricted in the center, for holding 3 g. of magnesium turnings in the upper portion, and (4) two pieces of glass tubing connected so as to fit into a rubber stopper which closes the neck of the flask and also receives the lower end of the condenser tube.

PROCESS. *Formation of Magnesium Benzoate.* Mix a suitable amount of the sample, diluted if necessary, with an excess of *40% sodium chloride solution*, add *phosphoric acid* to acid reaction, then 2 ml. in excess. Place a boiling tube in the flask, connect with the reflux condenser, and boil for 3 hours. Disconnect the flask, dissolve the magnesium benzoate formed in the test tube with hot water which also serves to rinse the condenser tube and the tubing attached to the stopper. A small beaker immersed for 10 minutes in a casserole of boiling water serves well for dissolving the benzoate from the turnings.

Oxidation. Filter the solution through glass wool and make strongly alkaline with about 20 drops of *40% sodium hydroxide solution.* Cool to 40 to 50° and oxidize with *saturated potassium permanganate solution* until the pink color persists for some minutes, then destroy the excess of permanganate by adding *sodium sulfite crystals* until the solution is clear brown. Add a few drops of *sulfuric acid;* this usually leaves the solution clear and colorless.

Ethyl Acetate-Naphtha Extraction. Saturate the solution with *sodium chloride* and extract with a *1 + 1 mixture of the two solvents.* Evaporate the solvents overnight at room temperature in a current of dry air, until no acid odor is evident.

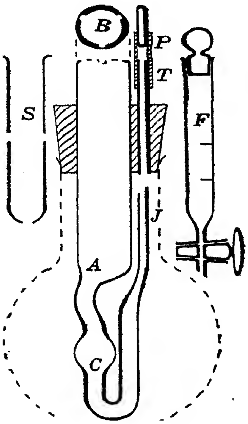
Titration. Dissolve in *ethanol* and water, add 1 or 2 drops of *phenolphthalein* and titrate with *0.02 N alkali.*

Leather Ferric Alum Steam Distillation Test.[8] The benzoic acid separated from the sample by sulfuric acid is steam-distilled into sodium hydroxide solution from which in turn it is liberated by acid, extracted with ether, and qualitatively examined. Leather (Manchester, England) states that as little as 1 mg. may be detected.

APPARATUS (Fig. 82). To a wide-mouth 250-ml. *flask* is fitted, through an easily removable rubber stopper, a special *U-tube* (*A*) and a *short tube* (*T*) (4 mm. inside diameter) closed by a *glass plug* (*P*) in a rubber tube connection. The large limb of the U-tube is 2 cm. in diameter and 10 cm. long, with three indentations (*B*) in the slightly inturned rim above and a 1.5-cm. bulb below; the narrow limb is narrowed to 2 mm. inside diameter and is cut off at a 45° angle at *J*. The thin-walled splash guard (*S*), 7.5 cm. long and 1.4

cm. outside diameter with holes in the bottom and sides, is suspended in the U-tube. The small cylindrical separatory funnel, with a capillary tap 1.5 mm. inside diameter, has a capacity of 4 ml. and graduation marks at 1 and 2 ml.

Process. Place 10 g. of the sample in the flask, add 75 ml. of *saturated brine*,[9] 1 ml. of *10% sulfuric acid*, and about 0.5 g. of coarsely powdered *pumice*. Attach the stopper carrying the glass parts, drop 0.5 ml. of approximately *1.0 N sodium hydroxide solution* into *A* so that it runs down into the *U*-bend, introduce the splash guard (*S*), and close *T* with the plug *P*. Heat the flask to boiling on iron gauze with a Bunsen flame and boil 3 minutes with a loss in weight of about 10 to 12 g. with little condensation.



Courtesy of Analyst 1931, **56**, *300*

Fig. 82. Leather Benzoic Acid Apparatus.

Remove stopper and attached parts, rinse with a jet of water, lift out the splash guard, and empty the U-tube through *J* into the separatory funnel (*F*). Add to the liquid 2 drops of *50% sulfuric acid* and 1 ml. of *ether*. Shake well, run off the aqueous layer, and wash the ether 3 times by shaking with an equal volume of water, then draw off the aqueous solution. Add to the ether solution a drop of *ammonium hydroxide*, noting if a turbidity, indicating 100 γ/g. of benzoic acid, appears.

Apply the following confirmatory test. Shake the separatory funnel well and draw off the aqueous layer into a hollow microscopic slide. Evaporate just to dryness over a covered water bath, cool, and dissolve the residue in a drop of water added from a platinum wire loop 5 mm. in diameter, then add at the edge of the drop from a loop 3 mm. in diameter a small drop of *7.5% ferric ammonium sulfate solution*. The presence of benzoic acid is indicated by the formation of a zone of an opaque buff precipitate of ferric benzoate.

LaWall and Bradshaw Salt–Chloroform Volumetric Method.[10] The method, based on observations of Moerck,[10'] has been improved by Bigelow and Dunbar.

Dunbar Modification.[11] In the form now official the technique is as follows.

Process. *Solution*. Place 150 ml. or 150 g. of the sample in a 500-ml. volumetric flask, add enough *pulverized sodium chloride* to saturate the water in the sample, and make alkaline to *litmus paper* with *10% sodium hydroxide solution* or *1 + 3 milk of lime*. Dilute to the mark with the *saturated salt solution*, shake well, and filter after allowing to stand 2 hours with shaking. For catsup add 15 g. of *pulverized salt*, then 150 ml. of the *saturated salt solution*, neutralize, and fill to the mark with the *saturated salt solution;* for jams, jellies, marmalades, and preserves, digest with 300 ml. of the *saturated salt solution* before adding 15 g. of *pulverized salt*, neutralizing, and making to the mark. Dealcoholize in slightly alkaline solution 250 ml. of cider and other alcoholic liquors. Wash 50 g. of salted or dried fish into a volumetric flask and treat as above, using, however, 30 g. of *salt*.

Extraction. Pipet 100 to 200 ml. of the filtrate into a separatory funnel, neutralize to

litmus paper with *1 + 3 hydrochloric acid* and add 5 ml. in excess. (Ignore a precipitate of proteins formed with fish.) Extract with 70, 50, 40, and 30 ml. of *chloroform*, shaking with a rotary motion to avoid an emulsion. If an emulsion forms, break it up by means of a glass rod, by drawing off into a second funnel and giving one or two sharp up-and-down shakes, or by centrifuging. Avoid drawing off any of the chloroform layer, thus obviating washing of the chloroform extract.

Transfer the extract to a porcelain dish and evaporate at room temperature in a current of dry air or first distil slowly in an Erlenmeyer flask to one-quarter the volume and then transfer to the dish for the final evaporation. Finally dry the residue over sulfuric acid or, in the case of catsup, until no acetic acid is evident.

Titration. Dissolve the residue in 30 to 50 ml. of *neutral (to phenolphthalein) ethanol*, add about 1/4 its volume of water, and 1 to 2 drops of *phenolphthalein indicator*, and titrate with *0.05 N sodium hydroxide solution*.

CALCULATION. Use the following formula: 1 ml. of 0.05 *N* sodium hydroxide solution = 0.0072 g. of anhydrous sodium benzoate.

SALICYLIC ACID

Ferric Chloride Test. PROCESS. *Preliminary Treatment.* Fruit sirups, non-alcoholic beverages, and many other liquids need no preliminary treatment. In the official A.O.A.C. method, emulsions are avoided by mixing 100 ml. with 5 g. of *sodium chloride*, diluting with *ethanol* to 250 ml., shaking vigorously, and filtering. The filtrate is rendered alkaline to *litmus* with *sodium hydroxide solution* and evaporated to 1/3 the original volume. Alcoholic liquids (200 ml.) are evaporated in like manner without addition of the salt. Solids and semi-solids (50 to 200 g.) are diluted to 400 ml. with water, mixed with 2 to 5 g. of *calcium chloride*, and rendered alkaline to *litmus*, then made up to 500 ml. and filtered.

Extraction. Render a suitable amount of the solution or the original sample distinctly acid with *hydrochloric acid* or *sulfuric acid* and shake with an equal volume of *ether*. Centrifuge if necessary to remove any emulsion. Draw off the aqueous layer, wash the ether solution with water, and evaporate to dryness on the water bath.

Color Formation. Add to the residue a drop of *0.5% ferric chloride solution*. A violet color appears if salicylic acid is present.

FORMALDEHYDE

Numerous tests have been devised for detecting formaldehyde and several questionable quantitative methods (including the Romijn method)[12] have been employed for determining the residual amount in the food, which, however, is far less than was originally added. Schiff's reagent (a 0.1% solution of fuchsin containing 2% of saturated sodium hydrogen sulfite solution and 1% of hydrochloric acid), when added to a solution or distillate containing any aldehyde gives a pink color of value in conjunction with the more specific method.

The first four of the following tests are official and are arranged in the order given in *Official and Tentative Methods of Analysis*, a second official phenylhydrazine-ferric chloride method and the phloroglucinol test, both of early origin, are here omitted, whereas the Barbier and Jandrier test is added.

Arnold and Mentzel Phenylhydrazine-Ferric Chloride Test.[13] To 5 g. of meat or fat, 10 ml. of milk, or 10 ml. of distillate from a solution rendered acid with *phosphoric acid*, add 0.03 g. of *phenylhydrazine hydrochloride* and 4 or 5 drops of *1% ferric chloride solution*, then slowly, with agitation in a bath of cold water, 10 to 12 drops of *sulfuric acid*. A red color indicates formaldehyde.

Hehner Iron-Acid Test.[14] To 5 or 10 ml. of milk or 5 ml. of a distillate obtained as above mixed with an equal volume of milk, add commercial *sulfuric acid*, or pure acid to

which has been added a trace of *ferric chloride*, by carefully pouring down the sides. A violet color indicates formaldehyde.

Leach Iron-Acid Test.[15] This differs from the Hehner test merely in that the milk or the distillate is heated to 80 to 90° in a porcelain casserole directly over the flame with 10 ml. of *hydrochloric acid* containing 2 ml. of *10% ferric chloride solution* per liter.

Rimini Phenylhydrazine-Nitroprusside Test.[16] To 20 ml. of milk or of the distillate obtained as above, add 1 ml. of *4% phenylhydrazine hydrochloride solution* and 4 drops of freshly prepared *0.5% sodium nitroprusside solution*, followed by several drops of *10% sodium hydroxide solution*. A blue or, if dilute, green color indicates formaldehyde. In the absence of formaldehyde, or in its presence on standing, or in the presence of acetaldehyde or benzaldehyde, a red color appears.

Barbier and Jandrier Gallic Acid Test.[17] Williams and Sherman [18] endorse this method. Mix 5 ml. of the distillate (see above) with 0.2 to 0.3 ml. of *saturated ethanolic gallic acid solution* and pour into 3 to 5 ml. of *sulfuric acid* in a test tube. A green zone at the juncture of the liquids indicates formaldehyde.

Formic Acid

See Organic Acids Part I, C6*b*.

Sulfur Dioxide

Direct Iodometric Method. See Part II, F1, under Organic Volatile Acids.

Carbon Dioxide Distillation-Barium Sulfate Gravimetric Method. Apparatus. (Fig. 83.) A *train* consisting of (1) a 250-ml. salt-mouth bottle (*M*) containing pieces of marble and provided with a separatory funnel (*F*) for introducing hydrochloric acid (sp. gr. 1.125), (2) a salt-mouth wash bottle (*W*) containing dilute sodium hydroxide solution to remove any sulfur dioxide from the gas, (3) a distilling flask (*S*), (4) an upright condenser (*C*), and (5) an Erlenmeyer flask (*R*) serving as a receiver. The gas entering *W*

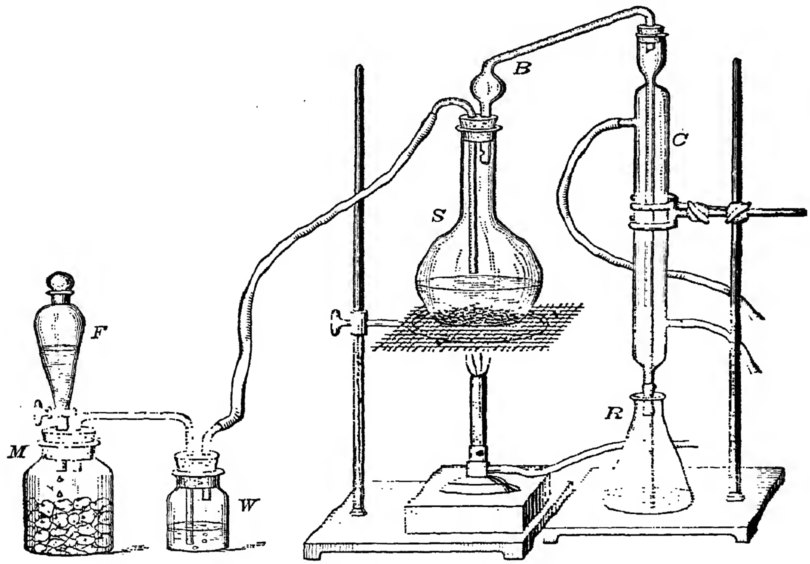


Fig. 83. Sulfur Dioxide Apparatus.

and *R* through *C* is delivered below the liquid, that entering *S*, just above the surface. *B* and *C* are the same as used for the Reichert-Meissl and the Polenske methods (see Part II, B2).

PROCESS. *Sulfur Dioxide Distillation.* Weigh on a balance accurate to 0.5 g. 50 g. of a solid substance or sirup (e.g., Hamburg steak, sausage, dried fruit, molasses, etc.) or 50 ml. of a liquid (e.g., wines, fruit juice). Transfer to flask *S*, add 200 ml. of water, and introduce the stopper with connecting tubes. Place enough water in the receiving flask *R* so that the delivery tube dips below the surface and add *bromine water* sufficient to impart a distinct yellow color.

Partially open the stopcock of *F* and allow *10% hydrochloric acid* to deliver drop by drop on the *marble*. The flow of carbon dioxide should be uniform and at a moderate rate, as shown by the escape of the bubbles through the liquid in *W*. After running a few minutes to insure the removal of all air, remove the stopper with tubes from *S* and without delay introduce from a pipet 5 ml. of *20% phosphoric acid*. The carbon dioxide gas, being heavier than air, does not escape from the flask. Close the flask immediately, bring to boiling with a Bunsen flame, and continue the boiling until the distillate measures about 150 ml. If the yellow color of the liquid in the receiver disappears, add more *bromine*, repeating if necessary.

Oxidation. The carbon dioxide prevents oxidation of the sulfur dioxide, which would take place in the air, until it comes in contact with the bromine, where it is converted into sulfuric acid.

Bromine Removal. After the distillation is complete, boil the distillate until the excess of bromine is removed, as shown by the odor, remove to a beaker, rinsing with water, and dilute further to about 250 ml.

Determination as Barium Sulfate. Add 1 ml. of *hydrochloric acid*, heat to boiling, and add *barium chloride solution* drop by drop until the precipitate of barium sulfate no longer forms. Allow to stand in a warm place overnight or longer. Prepare a porcelain Gooch crucible with a compact mat of amphibole asbestos about ¼ inch thick, ignite at bright redness, cool in a desiccator, and weigh. After weighing the Gooch crucible, place it again in the filtering apparatus, apply suction, decant the liquid from the precipitate of barium sulfate into the crucible, and finally transfer the precipitate to the crucible by means of a stream of hot water from a wash bottle. Remove any adhering barium sulfate from the beaker, using a "policeman." Wash about five times, nearly filling the crucible each time and allowing one portion to run through before adding another. Dry at a low temperature on a piece of asbestos paper heated by a small Bunsen flame, raise the heat cautiously, and finally ignite at dull redness for 3 minutes. Cool in a desiccator and weigh.

CALCULATION. Obtain the percentage, or grams per 100 ml., of sulfur dioxide (SO_2) in the material, using the following formula:

$$P = \frac{64.06 \times W \times 100}{233.43 \times 50} = 0.5489W$$

in which P is the percentage, or grams per 100 ml., of SO_2; 64.06 is 32.26 + 32, the molecular weight of SO_2; 233.43 is 137.37 + 32.06 + 64, the molecular weight of $BaSO_4$; and W is the weight of $BaSO_4$ found.

BORIC ACID

Turmeric Test. This time-honored test is the reverse of the test for turmeric. Usually it is sufficient to acidify a hot water extract of the sample or fluid milk with a few milliliters of *hydrochloric acid* per 100 ml., dip a strip of *turmeric paper* in the solution, and dry it on a watch-glass at room temperature. The characteristic brick-red color formed in the presence of boric acid changes with *ammonium hydroxide* to a dark blue-green. Addition of a drop of *acid* restores the red color.

A more delicate modification is to ignite

the sample, such as meat and fish, with an excess of *lime water*, evaporate to dryness, ignite at low redness, and make the test on a water extract of the ash, prepared by digestion on a water bath, after acidifying.

Gooch Methanol Distillation Gravimetric Method.[19] The fame of the inventor of the perforated crucible is enhanced by a series of inorganic methods devised during his career in the U. S. Geological Survey and Yale University.

APPARATUS. *Distillation Assembly*, consisting of (1) a 200-ml. distillation flask, provided with a double-bored rubber stopper carrying a small separatory funnel and a delivery tube, (2) a condenser, and (3) a 150-ml. platinum dish serving as a receiver.

PROCESS. *Methanol Solution*. Burn 400 or 500 g. of the sample with 10 g. of *calcium hydroxide*. Take up in cold *nitric acid*, precipitate the chlorine with *5% silver nitrate solution*, filter, wash, and make up to 500 ml. in a volumetric flask.

Distillation. Pipet 25 ml. of the solution into the distilling flask and connect with the condenser. Place in the platinum dish a suitable quantity of freshly ignited *calcium oxide*, ignite over the blast lamp, cool in a desiccator, and weigh. Arrange the removable delivery tube of the condenser so that it reaches to the bottom of the dish and add water to form a trap. Add to the contents of the distilling flask through the separatory funnel 10 ml. of *methanol* and distil in a paraffin bath at 140° with constant stirring of the contents of the dish to prevent escape of the boron. Repeat the distillation five times, using 12-ml. portions of *methanol*.

Ignition. Finally evaporate cautiously the contents of the dish, ignite, and heat at the same temperature as before so as to convert the lime into the oxide completely.

CALCULATION. Multiply the increase in weight due to boric oxide by 2.728 to obtain the corresponding weight of borax.

Thompson Mannitol Volumetric Method.[20] PROCESS. *Incineration*. Add to 100 g. of the sample in a platinum dish 1 to 2 g. of *sodium hydroxide* or sufficient to make the mixture strongly alkaline. Evaporate to dryness and ignite below redness until the organic matter is charred. After cooling, digest the residue with 20 ml. of hot water, acidify with *hydrochloric acid*, filter into a 100-ml. volumetric flask, and wash with hot water up to 50 to 60 ml. Return the filter and contents to the dish, add *lime water* to insure an alkaline reaction, and ignite to a white ash. Dissolve the ash in *1 + 3 hydrochloric acid*, filter into the 100-ml. flask and wash with a few milliliters of water. To the combined solutions, add 0.5 g. of *calcium chloride*, a few drops of *phenolphthalein indicator*, and *10% sodium hydroxide solution* to a light pink color. Dilute to the mark with *lime water*, mix, and filter through a dry paper.

Titration. Run into 50 ml. of the filtrate *1.0 N sulfuric acid* until the pink color disappears, then add *methyl orange indicator* and continue the titration until the yellow color changes to pink. Expel carbon dioxide by boiling for 1 minute, cool, and add carefully *0.1 or 0.2 N sodium hydroxide solution* until the liquid assumes a yellow tinge, avoiding an excess, then note the reading. Add to the solution, now containing the boric acid in a free state with no uncombined sulfuric acid present, 1 to 2 g. of *neutral mannitol* and a few drops of *phenolphthalein indicator*. Titrate again with the *standard sodium hydroxide solution* to a pink color, add a little more *mannitol*, and if the pink color disappears continue the addition until the color reappears. Continue the alternate additions of *mannitol* and *alkali* to a permanent end-point.

Neutral glycerol in volume equal to that of the solution may be substituted for the mannitol.

CALCULATION. Employ the following equivalents: 1 ml. of 0.2 N sodium hydroxide solution = 0.0124 g. of boric acid (H_3BO_3) or 0.007 g. of BO_3 or 0.0191 g. of $Na_2B_4O_7 \cdot 10H_2O$.

Fluorides

Modified Blarez Test.[21] Process. A. Silicates Absent. *Precipitation and Ignition.* Boil 150 ml. of the sample, or an extract of a solid food, and add during the boiling 5 ml. of *10% potassium sulfate solution* and 10 ml. of *10% barium acetate solution*. Collect the precipitate in a compact mass, centrifuging if necessary, remove to a small filter, and wash, then ignite the precipitate in a crucible.

Etching Test. Clean thoroughly a thin glass plate, heat, dip in a mixture of *cannuba wax* or *paraffin*, and cool. In the middle of the plate scratch a distinctive mark and place over the crucible to which has been added a few drops of *sulfuric acid*. Place over the plate a cooling device and heat the crucible for 1 hour at a temperature short of melting the wax. A distinct etching is indicative of fluorides.

B. Silicates Present. Mix a small quantity of *precipitated silica* with the precipitated barium fluoride and test for insoluble fluosilicates as described below.

Fluosilicates and Fluoborates

The following tests with little change have long been official. There is seldom need for their use.

Nivière and Hubert Tests.[22] Process. *Ignition and Extraction.* Evaporate 200 ml. of the sample with an excess of *lime water*, ignite, and extract with water containing sufficient *acetic acid* to decompose the carbonates, and filter. Ignite the residue and paper, then reextract with *1 + 2 acetic acid*, filter, and combine both extracts.

A. Fluosilicates. Ignite the second residue and filter, then mix with a little *silica* (SiO_2) and divide into two portions.

(*a*) Place one portion in a test tube and add 1 to 2 ml. of *sulfuric acid*. Attach a stopper connected with a U-tube containing a little water and heat in a beaker of water over a steam bath for 30 to 40 minutes. If fluorine is present, the silicon tetrafluoride will be decomposed and a gelatinous deposit will form on the walls of the tube.

(*b*) Add to the second portion, contained in a platinum crucible, a little *precipitated silica* and 1 ml. of *sulfuric acid*. Cover with a watch-glass from which hangs a drop of water and heat at 70 to 80° for 1 hour, keeping the watch-glass cooled. During the decomposition of the silicon tetrafluoride, a gelatinous deposit of silica and an etched ring are formed.

B. Boric Acid (Fluoborates). On the combined filtrates from the first and second extraction make tests for boric acid. The presence of both hydrofluoric acid and boric acid indicates the probable presence of boron trifluoride. If, however, only silicon tetrafluoride is detected, repeat the test, omitting the silica, in which case the formation of the silica skeleton indicates fluosilicates.

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PART II

SPECIAL METHODS

A. CEREAL FOODS

The true cereals are monocotyledonous dry fruits of the grass family (*Gramineae*). Buckwheat, although dicotyledonous, is usually classed with the cereals because of the similarity of the endosperm and its bread-making properties. Sorghum (related to maize), teff of Africa, and the millets of parts of Europe and Asia are true cereals.

Microscopic Structure of the True Cereals (Figs. 84 to 89). Included with the cereal group is green foxtail (Fig. 94) which, although a weed seed, belongs in the same botanical group.

Fruit Coat (Pericarp). Normally there are present (1) *epicarp cells*, hairy in wheat, rye, barley, and oats; (2) *hypoderm cells;* (3) *cross cells* (transversely elongated); and (4) *tube cells* (longitudinally elongated).

Seed Coat (Spermoderm). Membranous, thin-walled elongated cells.

Perisperm. Of rubbery indistinct cell structure.

Endosperm. (1) *Aleurone cells*, one or more layers of thick-walled cells containing aleurone grains but no starch, and (2) thin-walled *starch cells*, largest in the center of the kernel.

Embryo. A plantlet with *leaves* (plumule), *root* (radicle), and *cotyledon* (scutellum), all starch-free, but rich in protein and fat.

Chaff. Oat and barley chaff are characterized by (1) an *outer epiderm* of elongated cells with zigzag walls, stiff and soft hairs, hair scars in pairs (one crescent-shaped), and remarkable stomata, (2) a *middle layer* of "spongy parenchyma" consisting in oats of cells with radiating arms and in barley with cells more nearly rectangular.

Diagnostic Characters. Identification is chiefly by the character of the chaff, hairs, cross cells, and starch grains.

Microscopic Structure of Buckwheat (Fig. 92) ***and Black Bindweed or Wild Buckwheat*** (Fig. 93). Like the cereals, the grain is a dry fruit. The *black hulls* (pericarp) of the triangular fruit are not suited for human food. Structurally they have little in common with the bran coats of the cereals. The *seed* proper is similar to that of the true cereals in that it consists of starchy *endosperm* and oily *embryo*. The *seed coat* (spermoderm) elements of the buckwheats are very different from those of the true cereals, but the *starch grains* are about the same size as those of oats and rice; they do not, however, occur in large oval aggregates.

Microscopic Structure of Corn Cockle (Fig. 95). Unlike all of the products described herewith, corn cockle, one of several weed seeds belonging to the pink family (*Caryophyllaceae*), is a true seed. Although rich in nutrients, it is avoided by both man and cattle because of the presence of saponin and possibly other constituents. The characteristic elements are the conspicuous warty *seed coat* with papillae and the very minute *starch grains* occurring as elongated aggre-

LEGENDS OF ILLUSTRATIONS ON THE FACING PAGE

Cells that are longitudinally elongated in the kernel are so arranged in the figures. The magnification is ×160. The tissues of the hulls of barley and oats are portrayed on the second page of illustrations.

Fig. 84. Wheat. Elements in surface view. Fruit-coat layers on dorsal side of kernel: *epi*[2] epicarp, *hy* hypoderm, *in* intermediate cells, *tr* cross cells, and *tu* tube cells; *epi*[1] epicarp with hairs at apex. Seed coat: *o* outer and *i* inner layers. *N* perisperm. Endosperm: *al* aleurone cells and *am* starch grains.

Wheat hairs generally have a narrower inner diameter than those of rye and barley and the epicarp, hypoderm, and cross cells are more sharply beaded. The intermediate cells belong to an abortive second layer of cross cells. The large lens-shaped starch grains are similar to those of rye and barley, but with an average diameter greater than in barley and smaller than in rye.

Fig. 85. Rye. Elements in surface view. Fruit-coat layers on dorsal side of kernel: *epi*[2] epicarp, *hy* hypoderm, *in* intermediate cells, *tr* cross cells, and *tu* tube cells; *epi*[1] epicarp with hairs at apex. Seed coat: *o* outer and *i* inner layers. *N* perisperm. Endosperm: *al* aleurone cells and *am* starch grains.

Note (1) the tendency toward the broad inner diameter of the hairs, (2) the swollen end walls of the cross cells, and (3) the large size of the starch grains.

Fig. 86. Hulled Barley Elements in surface view. Fruit-coat on dorsal side of kernel: *epi*[2] epicarp, *hy* hypoderm, *tr* cross cells, and *tu* tube cells; *epi*[1] epicarp with hairs at apex. *S* seed coat. *N* perisperm. Endosperm: *al* aleurone cells and *am* starch grains.

Characteristic are the double layer of non-beaded cross cells and the lens-shaped starch grains with diameters less than in wheat and rye.

Fig. 87. Maize (Corn). Elements in surface view. Fruit-coat layers on dorsal side of kernel: *epi*[2] epicarp, *hy* hypoderm, *tr* cross cells, and *tu* tube cells; *epi*[1] epicarp at apex and *epi*[3] epicarp at base of kernel. *S* seed coat. Endosperm: *al* aleurone cells and *am* starch grains.

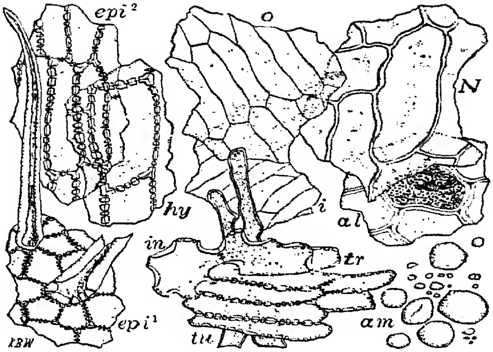
The absence of hairs on the kernel distinguishes corn from wheat, rye, barley, and oats, but hairs occur on the cob. The epicarp and hypoderm cells are more strongly thickened than in the other common cereals. The cross cells are irregularly tubular. The polygonal starch grains, each with a distinct, often branched, central cavity (hilum), are striking; similar starch grains occur in the sorghum group (kaffir corn, durrha, milo maize, etc.).

Fig. 88. Rice. Elements in surface view. Chaff: *ep*[1] outer epiderm with hair, *f* hypoderm fibers, *p* spongy parenchyma, and *ep*[2] inner epiderm with hair and stoma. Fruit-coat: *epi* epicarp, *hy* hypoderm, *tr* cross cells, and *tu* tube cells. *S* seed coat. *N* perisperm. Endosperm: *al* aleurone cells and *am* starch grains.

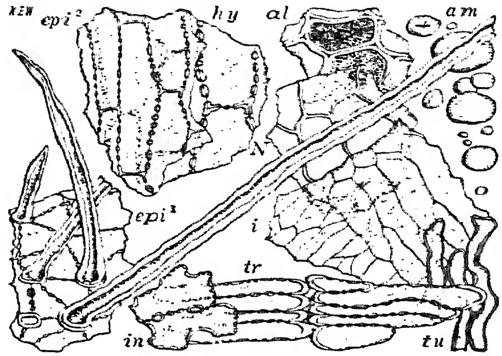
Rice hulls are characterized by the very thick, sinuous walls of the outer epiderm with stiff, pointed hairs. Hulled rice has no hairs. The delicate bran tissues are more or less transversely elongated. The small polygonal starch grains in aggregates are very similar to those of oats.

Fig. 89. Hulled Oats. Elements in surface view. Fruit coat: *epi* epicarp with hairs and *mes* spongy parenchyma. *S* seed coat. Endosperm: *al* aleurone cells and *am* starch grains.

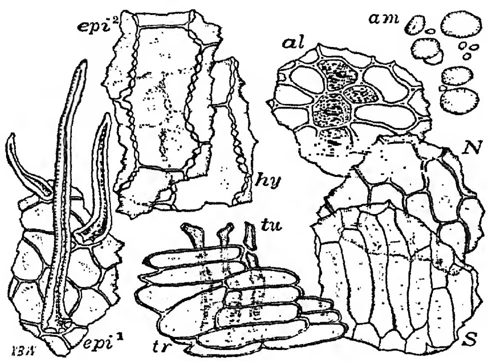
Of diagnostic value are the hairs, characterized by their relatively great length and the tapering toward the base, also the small polygonal starch grains, often forming oval aggregates.



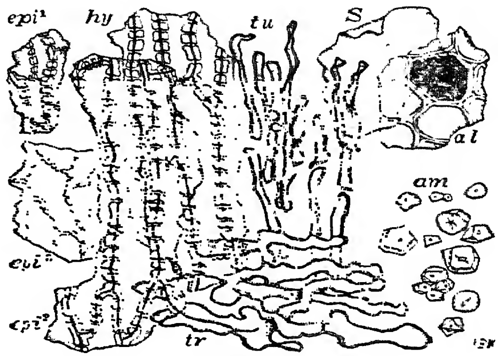
Wheat



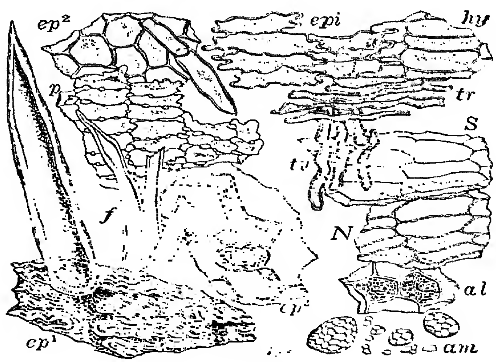
Rye



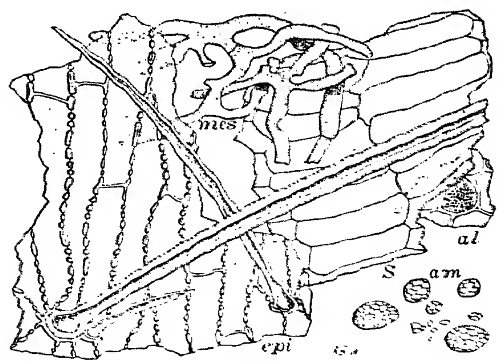
Barley



Maize



Rice



Oats

Figs. 84–89.

LEGENDS OF ILLUSTRATIONS ON THE FACING PAGE

These figures show the microscopic characters of barley chaff, oat chaff, buckwheat, and three weed seeds. The magnification is ×160, except for green foxtail which is ×300.

FIG. 90. Barley Chaff. Elements in surface view. t^1 hairs from bristle. t^2 hair from lodicule. Tissues on dorsal side of flowering glume: *aep*^{3} outer epiderm with long, round, and twin cells, *f* fiber, *fv* fibrovascular bundle, *p* spongy parenchyma, and *iep* inner epiderm with hair and stoma; *aep*^{2} outer epiderm at tip and *aep*^{1} outer epiderm at thin margin.

Note the large thick- and thin-walled hairs and the more or less rectangular form of the cells of the spongy parenchyma.

FIG. 91. Oat Chaff. Elements in surface view. Tissues on dorsal side of flowering glume: *aep*^{2} outer epiderm with long, round, and twin cells, *f* fiber, p^1 and p^2 spongy parenchyma, *fv* fibrovascular bundle, and *iep* inner epiderm with stomata; *aep*^{1} outer epiderm along marginal vein. *P* palet: right, at keel with long and twin cells and hairs; left, at edge with sawtooth hairs.

Of special diagnostic value are the sawtooth hairs and the radiating arms of the cells of the spongy parenchyma.

FIG. 92. Buckwheat. Elements in surface view. Calyx: *aep* outer epiderm with papillae and *st* stone cells. Fruit coat (hulls): *epi* epicarp, *f* fiber layer, *pig* pigment layer, and *end* endocarp. Seed coat: *aep* outer epiderm, *p* spongy parenchyma, and *iep* inner epiderm; *S* tissues at base of kernel. Endosperm: *al* aleurone cells and *am* starch grains.

Buckwheat hulls, with several characteristic tissues, are a waste product. The conspicuous elements of buckwheat flour are the spongy parenchyma of the bran and the starch grains; the latter are only slightly larger than those of oats and rice, but they are not combined as oval aggregates, although occasionally a few grains are attached end to end.

FIG. 93. Black Bindweed. *aep* outer epiderm of calyx in cross section. Fruit-coat elements in surface view: *epi*^{2} outer focus and *epi*^{3} deep focus of epicarp (*epi*^{1} in cross section), *hy* hypoderm, and *mes* mesocarp. Seed-coat elements in surface view: *ae* outer epiderm, *tr* cross cells, and *ie* inner epiderm. Endosperm: *al* aleurone cells and *am* starch grains.

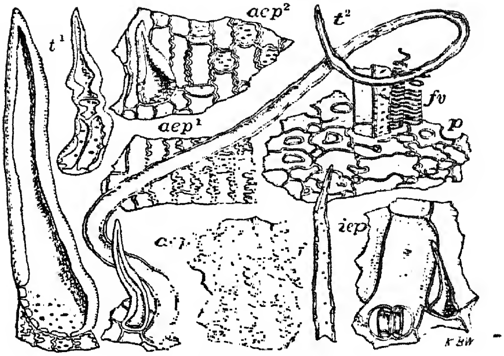
Of diagnostic value are the convoluted cells of the epicarp with warts. The starch grains are practically the same as in buckwheat.

FIG. 94. Green Foxtail. Elements in surface view. Outer epiderm of palet: *I* surface focus and *II* deep focus of immature, and *III* mature middle portion; *IV* mature edge. Fruit coat: *epi* epicarp, *tr* cross cells, and *tu* tube cells. *N* perisperm. Endosperm: *al* aleurone cells and *am* starch grains.

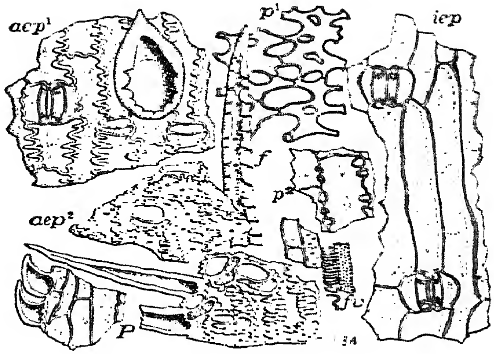
Note the compoundly wavy walls of the chaff and the polygonal starch grains.

FIG. 95. Corn Cockle. Elements in surface view. Seed coat: *aep* warty outer epiderm with papilla, *p* spongy parenchyma, and *iep* inner epiderm. Perisperm: *N* spiral reticulated outer epiderm and *am* starch grains. *E* epiderm of endosperm. *C* outer epiderm of cotyledon. *R* epiderm of radicle.

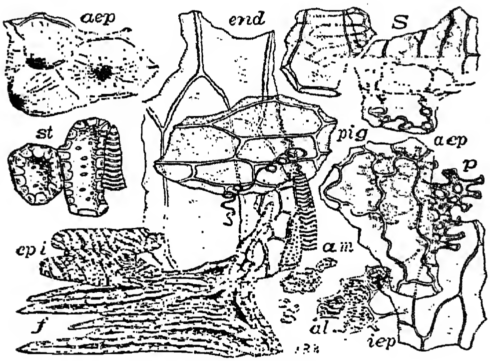
Characteristic are the finely warted outer epiderm of the seed coat with blunt or pointed papillae and the minute starch grains which are usually consolidated into elongated aggregates.



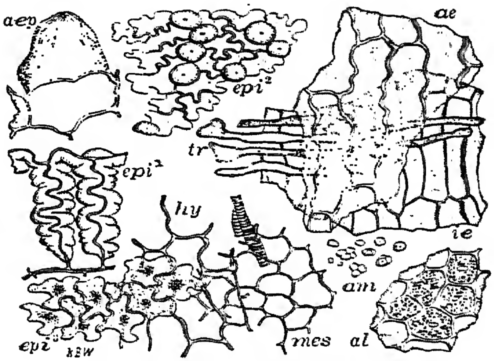
Barley Chaff



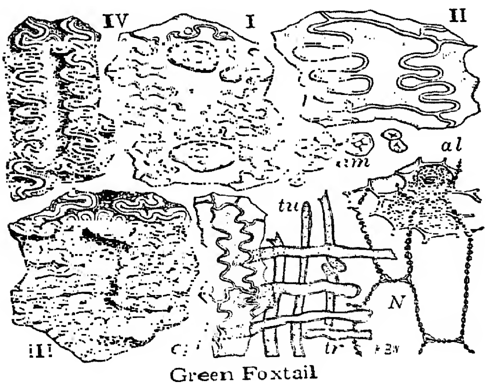
Oat Chaff



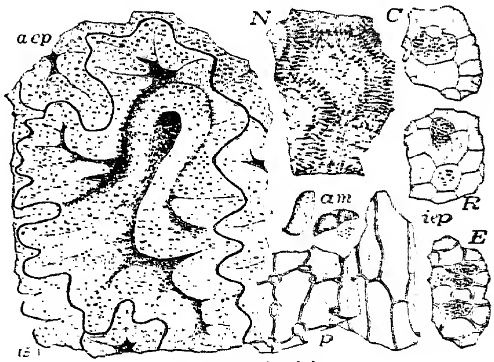
Buckwheat



Black Bindweed



Green Foxtail



Corn Cockle

Figs. 90–95.

Average Composition of Cereals and Cereal Products

	Water	Protein	Fat	Nifext	Fiber	Ash
	%	%	%	%	%	%
Wheat						
Kernels	10.62	12.23	1.77	71.18	2.36	1.82
Whole wheat flour	13.09	11.67	1.71	69.89	1.87	1.77
Patent flour	10.55	11.08	1.15	76.85		0.37
Clear flour	10.13	13.74	2.20	73.13		0.80
Red dog flour	9.17	18.98	7.00	61.37		3.48
Middlings	8.73	14.87	6.37	65.47		4.56
Bran	9.99	14.02	4.39	65.54		6.06
Germ	7.80	28.52	11.40	40.22	1.35	4.70
Shredded wheat	9.60	12.10	1.80	75.20	1.00	1.30
Durum wheat						
Kernels	11.10	14.60	....	....	....	1.57
Pastes (macaroni, etc.)	10.30	13.40	0.90	74.10	....	1.30
Rye						
Kernels	13.37	12.03	1.84	69.64	1.36	1.76
Flour	11.40	13.60	2.00	71.50	1.80	1.50
Bran plus middlings	10.84	16.69	3.52	.....	5.32	....
Barley						
Kernels	9.32	13.39	1.87	76.05	5.64	3.04
Pearl barley	11.50	8.50	1.10	77.80	0.30	1.10
Malt	7.76	10.76	1.56	71.04	6.18	2.70
Malt sprouts	7.06	27.17	2.40	.....	11.89	....
Corn						
Kernels	13.06	8.61	3.84	71.25	1.93	1.31
Hominy	11.80	8.30	0.60	79.00	0.90	0.30
Decorticated meal	12.50	9.20	1.90	75.40	1.00	....
Kaffir corn						
Kernels	12.36	12.11	3.63	68.08	2.39	1.43
Rice						
Kernels (polished)	12.85	7.52	0.38	78.05	0.47	0.73
Bran	10.67	11.29	9.97	46.02	10.95	11.00
Polish	10.63	10.94	7.02	63.34	2.62	5.45
Hulls	8.27	2.89	0.85	34.99	38.15	13.58
Oats						
Kernels	9.96	12.07	4.42	58.28	11.92	3.35
Rolled oats	8.40	16.04	6.79	65.57	1.45	1.75
Hulls	6.75	3.85	1.40	48.75	33.45	5.80
Buckwheat						
Kernels	12.62	10.02	2.24	64.43	8.67	2.02
Flour	13.60	6.40	1.20	77.90	0.04	0.90
Middlings	10.00	26.70	7.20	44.60	6.80	4.70
Hulls	6.50	7.80	1.40	47.10	33.60	3.60

Chemical Composition. The table herewith shows the general composition of cereals and their products as obtained by methods given under Part I, General Methods. These methods in essential details are those adopted by German chemists in the middle of the nineteenth century and are known as the Weende methods. Additional methods are given in the following pages classified under the head of (1) cereals, which in addition to the whole kernels, entire and ground, is made to include cereal products (other than flour), breakfast cereals, and by-products, (2) flour, or bolted meal, whole wheat flour being better classified as whole wheat, (3) oven products, and (4) alimentary pastes.

1. GRAIN; MEAL; BY-PRODUCTS

As stated above, the methods described in this section are not only for the whole cereals (or the equivalent ground cereals or meals) but also for a great variety of cereal preparations known as breakfast cereals, some for making porridge, others for immediate use. By-products, although forming a separate group, are included under cereals, since they are analyzed by the same methods. Methods for flour, oven products, and alimentary pastes are given in subsequent sections.

Many of the methods used for cereal analysis are applicable also to vegetable products in general and are accordingly described in Part I, General Methods.

Water

(Moisture)

See also Part I, C2*a*, and Part II, A2.

Brown and Duvel Oil Distillation Volumetric Method.[1] The method, as applied to whole cereals and oil seeds, may be carried out by the grain inspector without chemical training.

Apparatus (Fig. 96). The multiple apparatus consists essentially of a series of round-bottom side-tube flasks, heated in a chamber, a condensing tank, and graduates for collecting the distillates.

Reagent. *Hydrocarbon Engine Oil*, with open-cut flash point of 200 to 205°.

Process. *Distillation.* Weigh into the distilling flask a suitable quantity of the

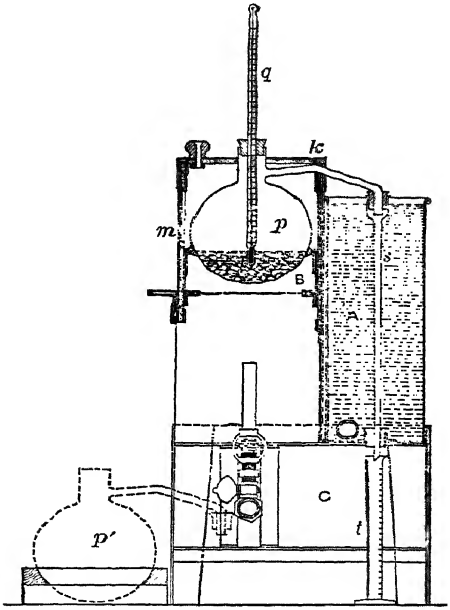
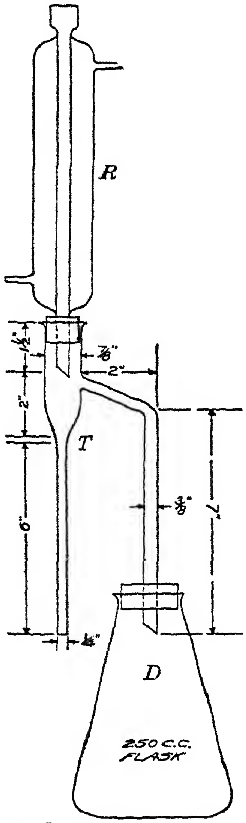


Fig. 96. Brown and Duvel Moisture Apparatus.

whole cereal or oil seed thus: 100 g. for wheat, rye, barley, corn (maize), unhulled rice, kaffir corn and related sorghums, flaxseed, or soy beans; 50 g. for oats or cottonseed. Add 150 ml. of *hydrocarbon engine oil*, introduce the rubber stopper carrying the thermometer so that the bulb of the thermometer dips into the oil mixture, and connect the side tube with the condenser tube. Heat the flask so as to reach in 20 minutes the appropriate temperature (corn, barley,

rice, kaffir, and cottonseed 190°, wheat 180°, rye and flaxseed 175°, soy bean 170°, oats 195°), then turn off the heat and wait until the water ceases to drip into the graduate.



Courtesy of Ind. Eng. Chem. 1925, 17, 147

FIG. 97. Bidwell and Sterling Moisture Apparatus.

Measurement. Carefully measure the number of milliliters of water in the graduate, thus obtaining without calculation (other than multiplication by 2 when the charge was 50 g.) the percentage of moisture.

Accuracy. The results are stated to agree closely with those by drying in a boiling water oven. Cook, Hopkins, and Geddes,[2] however, found that they are lower than those by drying in the vacuum oven, as are also those by drying in the air oven at 130°, after grinding in the Wiley mill. The standard error of prediction for the Brown and Duvel method and the air oven method was respectively hard red spring wheat 0.16 and 0.24, amber durum wheat 0.09 and 0.12, barley 0.12 and 0.20, and oats 0.13 and 0.20%.

Bidwell and Sterling Toluene Distillation Volumetric Method.[3] As originally devised at the U. S. Food Administration Laboratory the method was applied to a variety of products including molasses, honey, Karo sirup, jam, dried fruit, flour, butter, and dried milk, but as now used is listed under grain and stock foods. See also Part II, E3.

APPARATUS. *Distillation Assembly* (Fig. 97) consisting of a distillation flask (*D*) of Erlenmeyer form, a water trap or distilling tube receiver (*T*) with a narrow tube so graduated as to permit readings to 0.01 ml. and a sealed-in reflux condenser (*R*). Clean tube and condenser with oxidizing mixture, rinse well with water and ethanol, then dry thoroughly with heat.

PROCESS. *Distillation.* Cover the bottom of the distillation flask with dry *sand* and add about 75 ml. of *toluene* or sufficient to cover the sample, then add an amount of the sample sufficient to yield 2 to 5 ml. of water and assemble the apparatus. Fill the trap with *toluene* poured down through the condenser tube, heat to boiling, and distil at the rate of about 2 drops per second until most of the water has passed over, then increase the rate to 4 drops. When the distillation appears to be complete, rinse down the condenser tube with *toluene* poured in the top and continue the heating for a short time. If more water distils, repeat the rinsing. Detach any drops of moisture that may appear in the condenser tube with a delicate tube brush saturated with *toluene* and rinse with *toluene*.

Reading. Cool to the temperature at which the apparatus was calibrated, force down any adhering drops with a short piece of rubber tubing on a slender glass or metal rod, and read the volume.

CALCULATION. To obtain the percentage of water, multiply the volume of the water by 100 and divide by the weight of the charge.

Mohs and Mueller Rapid Drying Method.[4] These authors have devised a special drying chamber in which the drying of the sample can be finished within 13 minutes, half of the time being required for heating to 130°. The rapid drying is effected by a heating unit placed directly below the sample, a fan-ventilation system, and a device whereby the aluminum dish at the end of an inclined axis is rotated, thus constantly exposing new portions of the material to hot dry air. The results agree with those obtained by drying at 105° overnight.

Brabender Moisture Tester. The apparatus as exhibited at conventions and advertised [5] is designed for the automatic determination of moisture in general. One instrument replaces oven, balance, and desiccator. The percentage of moisture is read on a scale while the sample is in the oven. Ten determinations are made at a time with a sensitivity of 2 mg., the readings being to 0.05%.

PROTEIN

See Part I, C2*b* and C4*a*.

INDIVIDUAL PROTEINS

Separation and Determination of Cereal Proteins. The methods for the separation of the proteins into groups according to their solubilities are based on the results of investigations conducted by Ritthausen in Germany during the early seventies and by Osborne in the United States toward the close of the century. Teller, Snyder, and Chamberlain deserve credit for developing the details of conventional analytical processes.

The groups, as recognized by Osborne, are (1) *proteoses* (soluble in water, not coagulable by heat), (2) *albumins* (soluble in water, coagulable by heat), (3) *globulins* (insoluble in water, soluble in saline solutions), (4) *glutelins* or *glutenins* (insoluble in neutral solvents, soluble in very dilute alkalies and acids), and (5) *prolamins* or *gliadins* (insoluble in water, ethanol, and saline solutions, but soluble in 70% ethanol).

Commonly albumins and globulins are determined together and glutelins are obtained by difference. As pointed out by Chamberlain, there is more or less overlapping of the groups. For this reason, as well as because of the probability that some of the substances that have been isolated and named are not chemical individuals, it is well to use designations showing the solubility or insolubility, such as water-soluble proteins, salt-soluble proteins, ethanol-soluble proteins, water-, salt-, and ethanol-insoluble proteins, etc.

In the table below are given results obtained by Osborne and co-workers [6] in their investigation of the proteins of common cereals incidental to preparing the substances in the highest degree of purity.

The methods employed in obtaining these results were primarily designed to secure the proteins in the highest degree of purity and are much too intricate for ordinary analysis.

Teller Selective Solvent Method for Wheat Proteins.[7] The results are expressed in terms of nitrogen in the sample. The corresponding percentages of the proteins may be calculated by the factor 5.7.

PROCESS. *A. Non-Gluten Nitrogen.* To 5 g. of the pulverized sample contained in a 250-ml. volumetric flask, add 15 ml. of *brine (1% sodium chloride solution)* and shake well. Dilute nearly to the mark with the same solution and shake every 10 minutes for 1 hour. Add brine to the mark, shake, and allow to stand 2 additional hours. Decant

PROTEINS OF CEREALS (OSBORNE *et al.*)

	Spring Wheat	Winter Wheat	Rye	Barley	Maize
	%	%	%	%	%
Proteose	0.213	0.432	....	0.30	0.06
Albumin	0.391 *	0.359 *	0.43		....
Globulin	0.624 †	0.625 †	1.76 ‡	1.95	1.39
Glutelin	4.683 §	4.173 §	2.44	4.50	3.15
Prolamin	3.963 ‖	3.910 ‖	4.00	4.00	5.00

* Leucosin. † Edestin. ‡ Includes proteose. § Glutenin. ‖ Gliadin.

onto a filter, returning the filtrate, if cloudy, to the same paper.

Determine nitrogen in an aliquot of 50 ml. and subtract 0.27% to correct for gliadin soluble in the salt solution, thus obtaining the non-gluten nitrogen.

B. Edestin and Leucosin Nitrogen. To a 500-ml. Kjeldahl flask, add 50 ml. of the brine extract obtained above and 250 ml. of *ethanol*, shake thoroughly, allow to stand overnight, and collect the precipitate on a 10-cm. filter paper. Return the precipitate with filter to the flask and determine nitrogen in the joint proteins, correcting for nitrogen in the paper.

To separate the two proteins, coagulate at 60° the leucosin in a 50-ml. portion of the original brine extract, filter, and precipitate the edestin in the filtrate with *ethanol* as described above.

Determine nitrogen in the coagulum and the ethanol precipitate.

C. Amide Nitrogen. To a 100-ml. aliquot of the brine extract, add 10 ml. of *10% phosphotungstic acid solution*, thus precipitating all the true proteins. Allow to settle, filter, and determine the nitrogen in the filtrate.

The brine extract of bran and sprouted wheat may require more than 10 ml. of the phosphotungstic acid solution for complete precipitation of the proteins. In such cases, the filtrate should be tested by adding a small amount of the reagent.

D. Gluten Nitrogen. Subtract from the per cent of total nitrogen the per cent of non-gluten nitrogen or the sum of the percentages of edestin nitrogen, leucosin nitrogen, and amide nitrogen.

E. Gliadin Nitrogen. Heat for 2 hours on a water bath just below boiling 1 g. of the sample with 100 ml. of hot *75% ethanol* contained in a Kjeldahl flask, shaking during the first hour. Decant the hot supernatant liquid onto a filter, then heat the residue for 10 minutes with 7 successive portions of 25 ml. of hot *ethanol*, decanting onto the filter after each treatment.

Determine the nitrogen in the residue and filter paper, correcting for the nitrogen in the paper, or in the filtrate after distilling off the ethanol and drying.

From the per cent of nitrogen in the ethanol extract, subtract the per cent of amide nitrogen. The difference is gliadin nitrogen.

F. Glutenin Nitrogen. Subtract the per cent of gliadin nitrogen from the per cent of gluten nitrogen.

Chamberlain Selective Solvent Method.[8] The originator of this method first made a study of the method of Fleurent[9] as improved by Manget[10] in which gluten, prepared by washing dough with 0.1% sodium

chloride solution, is shaken with 0.2% potassium hydroxide in 70% ethanol until the gliadin is dissolved and the glutenin is emulsified, after which the glutenin is precipitated by carbon dioxide and an aliquot of the filtered solution is evaporated to dryness for the determination of gliadin. Having found that the Fleurent-Manget method gave too high results for gliadin and consequently too low results for glutenin, Chamberlain based a method on the data accumulated by Osborne and Voorhees [11] and the experience of Snyder [12] as follows.

A. Sample. Grind to pass through bolting cloth 90 to 100 meshes to the inch. Standard milled white flour is in suitable condition for analysis.

B. Salt-Soluble Proteins. Weigh out 4 to 6 g. of the sample into a 150- or 200-ml. flask, add 100 ml. of *5% potassium sulfate solution*, and shake thoroughly. Allow to stand for 18 to 24 hours with frequent shaking or, better, agitate in a mechanical shaker for 6 hours. Allow to settle, filter through a dry paper, and determine nitrogen in 50 ml. of the filtrate.

Multiply by 2 to obtain the amount of salt-soluble nitrogen in the sample and this last result by 5.68 to obtain the corresponding amount of salt-soluble proteins.

C. Ethanol-Soluble Proteins. Weigh 2 to 3 g. of the sample into a Kjeldahl flask, add 100 ml. of *70% ethanol*, and shake as in the determination of salt-soluble proteins. Allow to settle, filter, wash with *70% ethanol*, determine nitrogen in the entire filtrate, and calculate the proteins, using the factor 5.68.

D. Ethanol-Insoluble Proteins. Determine nitrogen in the residue from the ethanol extraction and multiply by 5.68 to obtain the proteins.

E. Salt-Soluble Proteins in Residue after Ethanol Extraction. Repeat the process of extracting the ethanol-soluble proteins, using, however, a smaller flask and rejecting the filtrate. Wash the residue back into the flask with 100 ml. of *5% potassium sulfate solution* and shake for the time and in the manner given under *C* above. Determine nitrogen in the entire filtrate and calculate the proteins, using the factor 5.68.

F. Glutenin. Subtract the amount of salt-soluble proteins in the ethanol-residue (*E*) from the ethanol-insoluble proteins (*D*).

G. Gliadin. Subtract from the amount of ethanol-soluble proteins (*C*) the amount of salt-soluble proteins (*B*) less the amount of salt-soluble proteins in the ethanol residue (*E*).

Moist and Dry Gluten; Alcohol-Soluble Protein; Gliadin; Salt-Soluble Protein; Albumin; Globulin; Glutenin; Amino Acids

See Flour, Part II, A2, below.

Alcohol-Soluble Acidity

The degree of acidity soluble in 80 to 85% ethanol serves as a test for spoilage of corn (maize) or corn meal.

Schindler Neutral Ethanol Volumetric Method.[13] The original method was employed by Winton, Burnet, and Bornmann [14] in an extensive study of the keeping qualities of corn meal.

Reagent. *Neutral Ethanol*, 85%. Distil 95% ethanol with quicklime and dilute to 85%.

Process. *Digestion.* Grind the sample to pass a 20-mesh sieve (approximately equivalent to 1-mm. holes) and weigh 10 g. into a 50-ml. glass-stoppered graduated flask. Fill to the mark with *neutral 85% ethanol* and let stand 24 hours with occasional shaking. Decant the supernatant liquid onto a dry pleated paper and pipet 25 ml. of the filtrate into a beaker.

Titration. Add to the aliquot 100 to 150 ml. of well-boiled distilled water a few drops of *phenolphthalein* and titrate with *0.05 N*

potassium hydroxide solution. Make a blank titration on the water and ethanol and introduce the necessary correction.

Calculation. Express the results in terms of milliliters of normal alkali per kilo of meal without correction for the volume of the insoluble portion of the sample, which represents a plus error of 10%.

Besly and Baston Modification.[15] The technique differs from that of the original method in that (1) a double charge (20 g.) is used and compensated by a half-strength *standard alkali solution* and (2) a measured amount (100 ml.) of *80% ethanol* is pipetted into an 8-ounce (250-ml.) bottle, thus reducing the error, which in this case is minus to the extent of about 2% of the result.

Fat

See Part I, C2*c*.

Fat Acidity

Zeleny and Coleman Benzene-Ethanol Volumetric Method.[16] The method was developed at the laboratory of the U. S. Bureau of Agricultural Economics, Washington. Since the acidity of grain due to spoilage is largely located in the germs and consists in large part of fatty acids, a determination made on an extract obtained with a fat solvent seems more logical than those made on ethanol solutions. It is also more rapid and reliable than the damaged kernel method based on the determination of the percentage of damaged kernels.

A. Process for all Cereals and Flour. See Part II, A2 below.

B. Rapid Process for Corn. *Fat Extraction.* Weigh 20 g. (±0.01 g.) of the sample into a 100-ml. glass-stoppered bottle, add exactly 50 ml. of *benzene*, stopper, and shake for a few seconds, then loosen the stopper to release the pressure and replace. Shake for 30 minutes in a mechanical shaker or 45 minutes by hand. Tilt, allow to settle for at least 3 minutes, and decant onto a pleated paper, covering the funnel to minimize evaporation while collecting exactly 25 ml. in a volumetric flask. Transfer the aliquot to a 250-ml. flask, refill the volumetric flask with *ethanol* containing *0.04% phenolphthalein*, and add to the 25 ml. of extract.

Titration. Using a color standard as under A, titrate with *standard 0.0178 N potassium hydroxide solution* to a distinct pink (white corn) or orange-pink (yellow corn) color.

Calculation. As under A.

Carotenoids

See also Part I, C10.

The carotenoids of yellow Indian corn (maize) of provitamin potency are carotene and cryptoxanthin, whereas zeaxanthin of the xanthophyl group is devoid of action. Carotene plus cryptoxanthin is determined jointly by the Clark and Gring method, α- and β-carotene separately by the Buxton method, and 5 carotenoids by the Fraps and Kemmerer method. See also Shrewsbury, Kraybill, and Withrow Method for α- and β-carotene and other methods for carotenoids in Part I, C10, under Carotenoids.

Carotene and Xanthophyl

Clark and Gring Spectrographic Method.[17] The authors publish from the University of Illinois.

Apparatus. *Spectrograph.* A Bausch & Lomb medium-sized quartz spectrograph or a Zeiss instrument of comparable construction fitted with a slit regulated in width by a micrometer screw. An under-water spark lamp described by Gring and Clark [18] or other suitable lamp. Absorption cell of Pyrex glass tubing 14 mm. wide and 20.4 mm. between the inner surfaces of the 1 mm. thick end covers of polished vitreous silica. The cell is provided with a side tube of 4-mm. Pyrex glass 60 mm. long.

PROCESS. *Sample.* Grind 50 g. of the corn for 12 to 20 hours in a 20-cm. flint ball mill, so as to pass an 80-mesh sieve, displacing the air by *solid carbon dioxide.* Store in *carbon dioxide gas* in a dark place at 4° until needed.

Extraction. Digest 10 g. of the sample at room temperature with *methanol* and centrifuge, repeating the operations, with 5 portions, until a solution of about 120 ml. is obtained.

Saponification. Add *solid potassium hydroxide* sufficient to make a 5 to 7% solution and saponify any xanthophyl esters by digesting for 2 to 3 hours.

Separation of Carotenes and Cryptoxanthin from Xanthophyl. Shake the saponified solution with *naphtha* in a separatory funnel and separate, repeating the addition, shaking, and separation until the extraction is complete. Wash the naphtha extract with *10% potassium hydroxide in 90% methanol,* to remove the last traces of xanthophyl, and finally with water until alkali is removed.

Evaporate the naphtha fraction to dryness in a vacuum desiccator at room temperature or lower to eliminate possible emulsion. Take up the solid mixture of carotenes and cryptoxanthin in a definite volume of *naphtha.* Reserve for the examination of the absorption spectra.

Purification of the Xanthophyl Fraction. Follow a procedure similar to that used for the other fraction. Dilute the alkali solution with 2 volumes of water, cool, and shake with *diethyl ether* in portions until the color is completely dissolved. Discard the methanol-water layer. Wash the combined xanthophyl fractions with water until the alkali is removed and evaporate to dryness in a vacuum desiccator. Take up the residue in *ethanol* and make up to a definite volume. Remove aliquots for the examination of the absorption spectrum.

Spectrographic Analysis. (1) Prepare a photographic plate showing several exposures of the carotene-cryptoxanthin absorption spectrum in duplicate or triplicate at different concentrations and, for comparison on the same plate, exposures of the absorption spectrum of the standard carotene (or cryptoxanthin) solution taken through diminishing widths of the slit, which is equivalent to diminishing the concentration and also prepare a corresponding plate for xanthophyl; (2) match the degree of density at 4500 Å of the unknown with one of the known by means of the microphotometer which, by passing the spectrographic plate transversely over the spectrogram at a uniform rate converts transmitted rays into corresponding degrees of electromotive force as measured by a galvanometer; and (3) plot a curve showing as abscissa the ratio $(s-f)/(b-f)$ in the microphotometer traces, and as ordinate the intensity I obtained by subtracting log I (light intensity) from I_0 (initial intensity). The b level is used as a base line for making other measurements; tracings are made with no light falling on the thermopile and correspond to the galvanometer rest point. The f level is reached while the light passes through a region of fogging (background) on the plate. The s level is produced while a spectrogram is intercepting the light beam directed on the thermopile.

CALCULATION. By the formula

$$\log I_0 - \log I = ksc$$

calculate the value of k (extinction coefficient) when c (concentration in gammas) is known as in preparing the standard, and of c when k is known as in an actual analysis, s the cell thickness being known in both cases.

For convenience, k is evaluated when s is expressed in millimeters and c in γ (10^{-6} g.)/ml.; thus k for β-carotene at λ 4500 $\pm$ 10 Å was found equal to 2.1×10^{-2} and k for xanthophyl, also at λ 4500 $\pm$ 10 Å, was 6.4×10^{-3}.

EXAMPLE. The following example given by Clark and Gring illustrates the calculation of carotene plus cryptoxanthin in a sample

of yellow corn. A diagram of the absorption spectrum of a particular sample shows the microphotometer trace made across the plate at λ 4500 Å. Another diagram shows the ratio $I : (s - f)/(b - f)$ as obtained from this same microphotometer trace of the "standard" spectrograms. Measurements of the three traces from absorption spectrograms of the sample gave $(s - f)/(b - f)$ values of 0.57, 0.55, and 0.57. These are evaluated in terms of I from the graph; then values for $(\log I_0 - \log I)$ are obtained equal to 0.48, 0.49, and 0.48 respectively. Substituting the value of k as 2.1×10^{-2}, and s as 20.4, and v as 10 ml. in $\log I_0 - \log I = ksc/v$, values of 11.3, 11.6, and 11.3 (aver. 11.4) γ per 10 g. are obtained for the three analyses of the sample.

More explicit explanations appear in Clark and Gring's paper and in the instructions accompanying the instruments.

Buxton Spectrophotometric-Chromatographic Method for Yellow Corn.[19] The features of the Buxton method, developed at the National Oil Products Co., Harrison, N. J., are (1) partition of carotene plus cryptoxanthin and zeaxanthin between heptane and methanol, (2) determination of carotene plus cryptoxanthin by the intensity of absorption band at 4500 Å, (3) removal of cryptoxanthin by adsorption on calcium carbonate, and (4) determination of the carotene thus purified by the intensity of absorption at 4500 Å and the cryptoxanthin by difference.

APPARATUS. *Modified Bausch & Lomb Visual Spectrophotometer* as used by Buxton and Dombrow [20] equipped with Hilger rotating sector disk and quartz biprism.

PROCESS. *Saponification.* Reflux for 1 hour with occasional agitation 20 g. of the finely ground corn with 200 ml. of *5% potassium hydroxide in methanol*, cool, allow to settle, and decant into 50 ml. of water contained in a separatory funnel.

Extraction. Extract the residue with five to six 50-ml. portions of purified technical *heptane.* Add the heptane extract to the methanol extract, shake the mixture well, and allow to settle. Remove the methanol layer and reextract with 50 ml. of *heptane.* Wash the combined heptane extracts free from alkali, testing with *phenolphthalein*, and xanthophyls by shaking thoroughly with 100-ml. portions of *90% methanol.* Reextract the first methanol washings with 50 ml. of *heptane.* Mix the heptane extract with a few milliliters of *isopropanol* and distil to small volume under reduced pressure in an atmosphere of *nitrogen gas.* Take up the residue in *heptane* and dilute in a volumetric flask to 50 ml. or other convenient volume with *heptane.*

Spectrophotometric Reading of Carotene and Cryptoxanthin. Determine the intensity of absorption at 4500 Å of the heptane solution with the visual spectrophotometer. Both carotene and cryptoxanthin show practically the same curve when plotted with wave lengths for abscissas and $E^{1\%}_{1\,\mathrm{cm.}}$ for ordinates, the maximum absorption being at 4500 Å, hence the spectrophotometric examination serves merely to determine both carotenoids together.

To obtain the γ of carotene plus cryptoxanthin for a 1% extract (G), use the following formula:

$$G = \frac{SE}{RC}$$

in which S is the screen factor, E the extinction coefficient for pure β-carotene (or cryptoxanthin) in heptane, R the reading expressed in centimeters, and C the concentration.

Chromatographic Separation of Carotene from Cryptoxanthin. Pour 25 ml. of the carotene-cryptoxanthin fraction on a modified Tswett column containing *powdered calcium carbonate* previously heated for 1 hour at 200 to 300° away from oxygen and cooled in an atmosphere of *nitrogen.* Develop the zones by washing with *heptane.*

All the carotene passes through the column, whereas all the cryptoxanthin is adsorbed by the carbonate.

Spectrographic Reading of Carotene. Read the carotene solution made up after chromatographing to a definite volume and calculate as before. Deduct the γ of carotene from the γ of the carotene plus cryptoxanthin to obtain the γ of cryptoxanthin.

Buxton reports 2430 at 4520 Å as a new extinction coefficient for cryptoxanthin.

EXAMPLES. Buxton gives results on 5 varieties of yellow corn showing 4.2 to 9.3 γ/g. of carotene plus cryptoxanthin, the average ratio of the two carotenoids being about 10 : 90 (carotene 6.9 to 14.4, cryptoxanthin 85.6 to 93.1%).

Fraps and Kemmerer Magnesium Oxide Chromatographic Method.[21] The method, originated at the Texas A. and M. College, is characterized by its simplicity and the number of carotenoids determined.

APPARATUS. *Jacketed Tswett Tube,* 15 to 20 cm. high and 5 to 8 mm. wide, constricted at the lower end.

Photoelectric Colorimeter.

A. PROCESS. *Extraction.* Reflux 25 g. of the finely ground yellow corn with *12% ethanolic potassium hydroxide solution* and extract the crude carotene with *naphtha* (b.p. 30 to 76.7°), as directed in the *A.O.A.C. Methods of Analysis.*

Color Reading. Dilute the crude carotene solution to 200 ml. in a volumetric flask and determine the pigment in terms of carotene with a photoelectric colorimeter.

Adsorption with Magnesium Oxide. Concentrate the solution to about 25 ml. and run through a column 10 cm. high of *magnesium oxide,* added in 0.2-g. portions and packed with a rod, cooling during the adsorption with ice water. Wash the column with naphtha until the bands separate (about 4 hours).

Extraction of Bands. Remove the 7 bands of pigment (3 and 4 together) while covered with naphtha and extract each with *98 + 2 naphtha-ethanol mixture,* then wash with water to remove the ethanol and, if cloudy, once with *dilute hydrochloric acid* (1 + 100). Dry over *anhydrous sodium sulfate* and dilute to a definite volume.

Color Reading. Determine the amount of pigment expressed as carotene with the photoelectric colorimeter.

EXAMPLES. A summary of results on groups as regards the content of carotene and cryptoxanthin of 18 and 4 varieties respectively is given in the table below. K-carotene is a new form of carotene. The numbers indicate the order in the column from top to bottom.

B. ABRIDGED PROCESS for pure carotene, cryptoxanthin, and impurities.

Gently pack 1 g. of *light magnesium carbonate* (instead of the oxide) in the tube with a cork tamp aided by suction and test by passing through it a solution of pure carotene (1.0 to 1.5 γ/g.), washing with *purified naphtha,* and determining the carotene in the filtrate. After washing for 30 minutes, the retention of carotene should not be over 5%.

Saponify 10 g. of the sample (A.O.A.C. Method), dilute the crude carotene to exactly 100 ml., and determine photometrically the crude carotene. Concentrate a 50-ml. aliquot *in vacuo* to 15 to 20 ml., place a few milliliters of *naphtha* on the column, apply suction, and, before all the naphtha has passed, pour in the solution of the unknown, then wash with *naphtha.* The carotene (including α-, β-, and K-carotene) passes through without forming a band. Make up to volume and determine pure carotene.

Separate the small bands of impurity at the top and the bottom of the column mechanically, elute with *naphtha containing 2% ethanol,* wash out the ethanol, and determine together. Separate also the cryptoxanthin and neocryptoxanthin bands, elute with *naphtha* and *ethanol,* wash out the ethanol with water, make up to volume, and determine together.

YELLOW CORN CAROTENOIDS (FRAPS AND KEMMERER)

	Crude Carotene	Composition of Crude Carotene					
		6 β-Carotene	7 α-Carotene	5 K-carotene	2 Crypto-xanthin	3 and 4 Neo-crypto-xanthin	1 Impurity *
	γ/g.	%	%	%	%	%	%
Group I							
Min.	1.3	23.9	0.8	3.5	33.3	5.9	0
Max.	8.0	35.8	6.1	7.9	53.3	20.2	8.6
Aver.	5.0	**30.7**	**4.0**	**5.7**	**42.2**	**15.8**	**1.5**
Group II							
Min.	3.7	16.8	0	3.4	39.7	21.1	0
Max.	7.4	22.6	3.1	6.9	53.2	29.0	4.2
Aver.	6.1	**19.9**	**1.1**	**5.3**	**48.7**	**24.0**	**1.1**

* When less than 1%, impurity is determined by difference.

THIAMIN

Jansen Ferricyanide-Thiochrome Fluorometric Method; Pyke Modification. See Part I, C10.

Johannson and Rich [22] have further modified the method for use in the analysis of wheat and its products.

Andrews and Nordgren Modification.[23] This modification is a simplification of the original method. By its use results agreeing with those by rat growth were obtained on certain cereal products.

RIBOFLAVIN

Andrews, Boyd, and Terry Taka-Diastase-Florisil Fluorescence Method.[24] In this method (General Mills, Inc.) the role played by the amylolytic enzyme is in the preparation of the extract rather than in the method proper, which combines features of the Hodson and Norris modification of the von Euler and Adler method for riboflavin and the Conner and Straub Decalso-Supersorb method for thiamin and riboflavin.

APPARATUS. *Pfaltz and Bauer Fluorophotometer*, with the 511-038 combination filter for incident light and No. 351 filter for fluorescent light.

PROCESS. *Extraction.* Autoclave 10 g. of patent or 5 g. of whole wheat flour with 90 ml. of water, cool to 50°, add 5 ml. of *6% taka-diastase solution*, and allow to stand 30 minutes. Make up to 100 ml. in a volumetric flask and centrifuge.

Prepare also an aqueous extract in the same manner, omitting the taka-diastase.

Florisil Adsorption. Pass a 20-ml. aliquot through a column of Florisil, wash the adsorbent with 5 to 10 ml. of water, and dry in an air current.

Elution. Use sufficient *20% pyridine solution in 2% acetic acid* to secure an eluate of 20 ml.

Fluorescence Reading. Pipet 14 ml. of the well-mixed eluate into the cell of the fluorophotometer, adjusted by means of the iris

diaphragm to yield a 25 scale division deflection of the galvanometer, for 0.1 γ/ml. sodium fluorescein solution, then determine the fluorescence (A). Under these conditions pure riboflavin solution (0.2 γ/ml.) gives a deflection of 27 scale divisions.

Determine also the fluorescence after adding 1 ml. of a solution containing 1.5 γ/ml. of pure riboflavin in *20% pyridine in 2% acetic acid* (B). Then stir in 10 to 20 mg. of *solid sodium hydrosulfite* ($Na_2S_2O_4$) to reduce the riboflavin and again determine the fluorescence (C).

Calculation. Obtain the riboflavin content (R) by the formula

$$R \quad \frac{A - 1.07C}{B - 0.934A} \times 0.1 \times \frac{100}{W}$$

in which W is the weight of the portion of the sample taken.

Precautions. Carry out all operations in a darkened room. Adjust the concentration of fluorescein (which may vary) to correspond approximately to that of riboflavin. Use only taka-diastase containing an insignificant amount of riboflavin. Avoid a too low pH, which necessitates extraction with dilute acid.

Smuts

As defined by the U. S. Grain Standards,[25] wheat or rye is regarded as smutty when the grain has an unmistakable odor of smut or contains balls or spores of smut in excess of an equivalent of 14 balls of average size weighing 0.01 g. and containing an average of 3,750,000 spores or 52,520,000 spores per 250 g. of grain.

Official Smut-Counting Method. Apparatus. *Compound Microscope*, magnifying 50 diameters.

Haemacytometer, with Neubauer or similar ruling.

Dropper, with end drawn to a fine capillary point.

Process. *Separation of Smut.* Pick out and weigh all smut balls and pieces of balls from 50 g. of the sample. Place the sample in a 500-ml. Erlenmeyer flask, add 100 ml. of water, stopper, and shake vigorously for 2 minutes. If the count is not to be made immediately, pour the smutty water into another flask.

Counting of Smut Spores. Place the cover plate over the grating of the newly cleaned haemacytometer so that it rests on the raised guides on each side of the grating. Agitate the smut suspension, draw a small portion into the dropper, and quickly, but carefully, let a small portion be drawn under the cover-glass by capillary attraction. Avoid an excess of water and bubbles. Count under the microscope the number of spores in nine squares in succession and note the total number of spores.

Calculation. Obtain the number of spores in 100 ml. of water-smut suspension and divide the number by 3.75×10^8 to obtain the grams of smut in the suspension. Add the quotient to the weight of the whole or broken smut balls previously determined. Calculate the total weight of smut per 250 g. If this exceeds 0.14 g. (0.05% by weight), grade the grain as smutty.

Sulfur Bleaching

Carroll Lead Sulfide Test.[26] Apparatus. *Erlenmeyer Flask*, connected by a tight-fitting cork stopper with a U-shaped delivery tube: short arm 5 cm. (2 in.); long arm 15 cm. (6 in.); cross arm 10 cm. (4 in.).

Process. Place 100 g. of the grain and 10 g. of *mossy zinc* in the Erlenmeyer flask, cover the grain with *1 + 4 hydrochloric acid*, quickly attach the cork and delivery tube, the short arm of which is loosely packed with absorbent cotton, and insert the long arm into a test tube three-quarters filled with *1% basic lead acetate solution*. If sulfur is present, a black precipitate of lead sulfide is formed.

2. FLOUR

U. S. Standards.[27] *Flour* (white, wheat, or plain, not durum) is ground from wheat, with provision for malted wheat or flour and up to 0.25% of malted barley flour and bolting through the U.S.B.S. 149 μ (No. 100) cloth. It contains not more than 15% of water and 0.05 $\times$ protein content + 0.35% of ash, dry basis. If bleached (p. 445), a declaration is required. *Enriched flour* contains thiamin 1.66 to 2.5, riboflavin 1.2 to 1.8, niacin or its amide 6 to 24, and iron 6 to 24 mg./lb. Addition of vitamin D 250 to 1000 U.S.P. units, calcium 500 to 2000 mg./lb., and germ up to 5%, also acidulation with $CaH_4(PO_4)_2$, irrespective of the minimum limit of calcium, are permitted. *Bromated flour* contains not more than 75 μ/g. of $KBrO_3$ and not less than 15% of protein, dry basis. *Self-rising flour* contains $CaH_4(PO_4)_2$ or $Na_2H_2P_2O_7$ and $NaHCO_3$ to yield 0.5% of CO_2. Standards for *phosphated flour*, *durum flour*, *whole wheat flour*, *crushed wheat*, *cracked wheat*, *farina*, and *semolina* are given.

Water

(Moisture)

See also Hydrogen Drying Method, Part I, C2*a*.

Spencer Loose Cover Vacuum Method.[28] Process. Weigh 2 g. of the flour or ground cereal into a tared and dried covered dish, loosen the cover, heat at 98 to 100° for 5 hours at a pressure not more than 25 mm. of mercury, tighten the cover, cool 20 minutes in a desiccator, and weigh.

Rapid Process. Heat at 130° in a ventilated air oven for 1 hour without the cover, replace the cover, cool in a desiccator for 20 minutes, and weigh.

The results are practically the same as those obtained by the vacuum method.

A.A.C.C. standard tables and charts for conversion of analytical data to a 14% water basis are supplied by H. L. Harris, University Farm, St. Paul 8, Minn., for 50 cents.

Protein

See 1, Grain; Meal; By-Products, above.

Gluten

The term gluten is often loosely used. It is not synonomous with protein and it is not a constituent of the wheat kernel, since it is formed by the combination of gliadin and glutenin during doughing. The proportion of the constituents varies in the gluten thus obtained and is dependent on the proportion in the grain and flour, the presence of other constituents, and the details of the process of preparation. No flour, other than wheat flour, yields a considerable amount of gluten and even wheat flour, when it or the grain has been damaged, may yield little or none.

Bamihl Test for Gluten.[29] This test, specially designed for detecting inferior grades of wheat flour in rye flour and later used for detecting wheat flour in buckwheat and other flours, also serves as a ready demonstration of the formation and properties of gluten.

As modified by Winton,[30] a coal-tar dye added to the water brings out strikingly the formation of the gluten and its affinity for the color.

Reagent. *Eosin Solution.* Dissolve 0.2 g. of water-soluble eosin in 1 liter of water.

Process. Weigh on a microscopic slide 1.5 mg. of the flour and add a drop of *eosin solution.* Place on white paper. Mix by rotating a circular cover-glass, held at an angle, with one edge on the slide in such a manner that neither the flour nor the water escapes beneath it, then allow it to drop and rub it back and forth until the gluten forms rolls which absorb the dye with avidity.

Dough-Washing Gravimetric Method for Moist and Dry Gluten in Wheat Flour.[31] Work up into a dough with a spatula in a cup

25 g. of flour and 15 ml. of water at room temperature, avoiding adherence to the cup. After 1 hour, knead in a stream of cold tap water over a piece of bolting cloth, conveniently held in place by an embroidery hoop, until the starch is no longer evident as a cloudiness in the liquid. Keep the gluten mass in cold water for 1 hour, then work with the hands to remove as much water as possible, make into a ball, and weigh in a tared flat-bottom metal dish as *moist gluten.*

Flatten the wet gluten so as to cover the bottom of the dish, dry for 24 hours in a boiling water oven, cool in a desiccator, and weigh as *dry gluten.* Repeat the drying if the presence of appreciable moisture is suspected.

The method yields concordant results only if duplicates are treated in exactly the same manner. A more satisfactory procedure is to determine nitrogen by the Kjeldahl method and calculate the protein, using the factor 5.70.

Salt-Soluble Protein, Albumin, Globulin, Gliadin, and Amino

Mitchell Brine Digestion Volumetric Method.[32] Process. *Brine Digestion.* Weigh 10 g. of the sample into a 500-ml. Erlenmeyer flask, add 250 ml. of *1% sodium chloride solution*, and allow to digest at room temperature for 3 hours with occasional shaking, then filter.

A. Salt-Soluble Nitrogen. Boil down 100 ml. of the filtrate to small volume in a Kjeldahl flask with 5 ml. of *sulfuric acid*, add 25 ml. more *acid*, and determine the nitrogen.

B. Amino Nitrogen. To a second 100-ml. portion of the filtrate, add 5 ml. of *20% phosphotungstic acid solution*, shake well, let settle, and decant on a filter. Wash slightly with water, evaporate the filtrate with 5 ml. of *sulfuric acid* in a Kjeldahl flask, and determine the nitrogen as above.

C. Albumin Nitrogen plus Globulin Nitrogen. Subtract the amino nitrogen from the salt-soluble nitrogen.

Blank. Make blank determinations and correct accordingly.

Calculation of Proteins. Employ the factor 5.70.

Olson Modification.[33] Process. *Brine Digestion.* To 10 g. of the flour, add 500 ml. of *1% sodium chloride solution*, shake at intervals of 5 minutes for 1 to 2 hours, then let stand in a cool place overnight and filter.

A. Salt-Soluble Nitrogen. Determine in 200 ml. of the filtrate.

B. Albumin Nitrogen. Pipet another 200-ml. aliquot of the filtrate into a beaker, boil down to about 20 ml., and slowly evaporate to dryness on a hot plate. Digest the residue with 100-ml. portions of *55% (by volume) ethanol*, filter, and wash the residue on the paper with *ethanol* of the same strength. Determine nitrogen in the residue and correct for nitrogen in the paper.

C. Globulin Nitrogen. In the filtrate from the ethanol treatment, precipitate the globulin with *phosphotungstic acid solution* and wash with water containing that acid. Determine nitrogen in the precipitate and paper and correct for nitrogen in a blank determination.

D. Amide (Amino) Nitrogen. Determine nitrogen in the filtrate from the phosphotungstic acid precipitation.

E. Gliadin Nitrogen. Subtract the sum of the nitrogen as albumin, globulin, and amide from the total salt-soluble nitrogen.

Crude Gliadin

Chamberlain 70% Ethanol Volumetric Method.[34] See Part II, A1, above.

In the modification adopted by the A.O.A.C. (tentatively) and the A.A.C.C., 4 g. of flour are shaken with 100 ml. of 70% ethanol, either at half-hour intervals for 5 or 6 hours, or continuously for 1 hour in a

shaking machine, before allowing to stand overnight. A deduction is made for the nitrogen found in a blank determination.

Snyder Polarimetric Method.[35] REAGENT. *Millon Reagent.* Dissolve a convenient amount of metallic mercury in an equal weight of fuming HNO_3 and dilute with an equal volume of water.

PROCESS. *Digestion.* Digest 15.97 g. of the sample with 100 ml. of *ethanol* (sp.gr. 0.90) for 3 hours with shaking at 30-minute intervals and let stand overnight.

Direct Polarization. Filter through a dry pleated paper and polarize the filtrate in degrees Ventzke in a 200-mm. tube.

Polarization after Precipitation with Millon Reagent. To exactly 50 ml. of the filtrate, add from a pipet 5 ml. of freshly prepared *Millon reagent*, shake, filter, and polarize the filtrate in a 220-mm. tube.

CALCULATION. Subtract the reading of the second polarization from that of the first and multiply the product by 0.2 to obtain the per cent of gliadin nitrogen, and this in turn by 5.70 to convert to per cent of crude gliadin.

SALT-SOLUBLE PROTEIN

Chamberlain Potassium Sulfate Volumetric Method.[36] Modified as to the weight of the charge, volume of the solvent, and time of digestion, the method, as adopted by the A.O.A.C. (tentatively) and by the A.A.C.C., is as follows.

PROCESS. Digest 6 g. of the sample with 100 ml. of *5% potassium sulfate solution* at room temperature for 3 hours, with shaking at 30-minute intervals, or, better, agitate at moderate speed in a mechanical shaker for 1 hour, and let settle for 30 minutes. Filter and determine nitrogen in 50 ml. of the filtrate.

CALCULATION. Deduct the amount of nitrogen found in a blank determination and multiply by 5.70.

GLUTENIN

Blish and Sandstedt Alkali–Methanol Volumetric Method.[37] As developed at the Nebraska Agricultural Experiment Station, the extraction is made with alkali and methanol and the glutenin is precipitated with acid.

Blish, Abbott, and Platenius Barium Hydroxide Volumetric Method.[38] The method depends on the solubility of all the nitrogenous matter but glutenin in barium hydroxide–methanol mixture.

PROCESS. *Digestion.* To 8 g. of the sample in a 200-ml. Kohlrausch flask, add exactly 0.2 g. of *powdered barium hydroxide* and 50 ml. of water. Digest for 1 hour at room temperature with frequent shaking and make up to 200 ml. with *96 to 99% acetone-free methanol*, then add exactly 5 ml. more to compensate for the volume of the flour. Mix well and filter through a well-packed cotton plug.

Nitrogen Determination. Without delay pipet 50 ml. of the filtrate into a Kjeldahl flask and determine the per cent of nitrogen.

CALCULATION. Multiply the per cent of nitrogen by 5.70 and subtract the product from the per cent of total protein obtained by the same factor, thus obtaining the per cent of glutenin.

NOTE. The A.O.A.C. also (tentatively) obtains glutenin by subtracting the sum of the potassium sulfate–soluble and ethanol-soluble nitrogen from the total organic and ammoniacal nitrogen and multiplying by 5.70.

AMINO ACIDS

Modified Sørensen Formaldehyde Volumetric Method.[39] REAGENT. *Buffer Solution, pH 8.0.* Mix 50 ml. of 0.2 *M* K_2HPO_4 and 46.85 ml. of 0.2 *M* NaOH.

PROCESS. *Extraction.* Shake well 20 g. of the sample and exactly 100 ml. of water in a 250-ml. Erlenmeyer flask and let stand 1 hour, shaking every 10 to 15 minutes. Cen-

trifuge, filter if turbid, and pipet 10 ml. of the extract into a 100- to 125-ml. Erlenmeyer flask.

Titration. Add to the aliquot 0.2 ml. of *0.02% phenol red indicator* and titrate to pH 8.0 with *N/14 sodium hydroxide solution* delivered from a micro buret calibrated to 0.01 ml., matching against the pH 8.0 buffer solution (A). Add 0.18 ml. of *phenol red indicator*, 8 ml. of *40% formaldehyde*, and again titrate to pH 8.0 (B). Make a blank determination on the formaldehyde, using 10 ml. of water instead of the flour extract (C).

CALCULATION. Obtain the amino nitrogen as milligrams per 100 g. (M) by the formula

$$M = B - (A + C)$$

NOTE. If desired, the solutions may be titrated electrometrically.

FAT

Ether Dry Extraction Method. The technique, employing a continuous flow extractor, is given in Part I, C2*c*.

Jacobs and Rask Ammonia-Ether-Naphtha Wet Extraction Method.[40] The method is essentially the Röse-Gottlieb method adapted to the determination of fat in flour.

PROCESS. Digest 5 g. of the flour in a 200-ml. Erlenmeyer flask on a steam bath for 2 minutes with a mixture of 10 ml. of *ethanol*, 2 ml. of *ammonium hydroxide*, and 3 ml. of water. Cool and digest with 3 successive portions of *ether*. Evaporate the combined ether extract to dryness on a steam bath and extract the fat from the residue with equal parts of *ether* and low-boiling-point *naphtha*. Evaporate the combined ether-naphtha extract in a tared beaker, on the steam bath, dry at 100° for 1 hour, cool, and weigh.

Hertwig Acid-Ether-Naphtha Wet Extraction Method.[41] The method differs from the Röse-Gottlieb-Patrick method in that the digestion is in acid instead of ammoniacal solution, but is similar to the Schmidt-Werner method. See Part II, G1.

PROCESS. *Acid-Ethanol Digestion.* To 2 g. of flour in a 50-ml. beaker, add 2 ml. of *ethanol* and stir so as to moisten the particles, thus preventing lumping. Add 10 ml. of *25 + 11 hydrochloric acid*, mix thoroughly, and digest for 30 to 40 minutes in a water bath at 70 to 80° with frequent stirring, then add 10 ml. of *ethanol*, and cool.

Ether-Naphtha Extraction. Transfer the acid mixture to a Röhrig or Mojonnier extraction tube, rinse the beaker with 25 ml. of *ether* in three portions, and shake well. Add 25 ml. of redistilled *naphtha* (b.p. below 60°) and shake again. Let stand until the upper liquid is practically clear, then draw off as much as possible of the fat solution, through a filter consisting of a pledget of cotton so packed in the stem of a funnel as to allow free passage of the solution, into a tared 125-ml. beaker flask containing bits of broken porcelain or glass, which together with a counterpoise has been dried at 100° and allowed to stand in the air to constant weight.

Repeat the extraction and filtration through the pledget, using, however, 15 ml. of each *solvent*, shaking after the addition of each and rinsing the tip of the spigot and funnel stem, as well as funnel, with a few milliliters of the *dry mixed solvents*.

Evaporate the ether slowly on the steam bath and dry the flask with fat and counterpoise to constant weight in the oven at 100°. The drying is complete in about 90 minutes and the cooling requires about 30 minutes.

Correct the weight for any matter obtained in a blank determination.

Grossfeld Acid-Trichloroethylene Wet Extraction Method.[42] The method as first proposed was designed for meat, cheese, and cocoa.[43] It resembles the Röse-Gottlieb and the Schmidt-Bonzynski methods, but employs as the solvent trichloroethylene, which is characterized by nearly complete insolubility in water, low volatility, high spe-

cific gravity, non-inflammability, and low cost. The results were found to agree with those by ether extraction.

PROCESS. *Acid Extraction.* Boil for 10 minutes 10 g. of the flour or meal or the air-dry bread in a 300-ml. round-bottom flask with 100 ml. of water and 25 ml. of *25% hydrochloric acid.* The foaming, which occurs at first, diminishes on boiling. Cool, add 5 ml. of *0.1% Congo red solution* and then *10% sodium hydroxide solution* to faint acid reaction. Filter through a dry fluted paper and wash twice with water. Allow to drain, then spread out the paper and insoluble matter on copper gauze, and dry.

Trichloroethylene Extraction. Cut up the filter paper and contents, place in a dry round-bottom flask, add exactly 100 ml. of *trichloroethylene* and reflux for 5 minutes, using an efficient condenser. Cool to room temperature and filter, keeping the funnel covered with a watch-glass.

Pipet, or measure in a pycnometer, 25 ml. of the filtrate into a tared 100-ml. Erlenmeyer flask. Evaporate the solvent, at first over the free flame, and finally for 1 hour at 105 to 110°, keeping the flask in an inclined position, and weigh.

FAT ACIDITY

Zeleny and Coleman [44] showed that as wheat and corn deteriorate in storage, the fat acidity increases rapidly, whereas phosphate acidity and amino acid acidity increase only during relatively advanced stages of deterioration.

Kozmina and Alakrinskaya Volumetric Method.[45] This method may be regarded as the precursor of the Zeleny method given next below.

PROCESS. *Extraction.* Place 20 g. of the flour in a filter thimble, insert in a 350- to 400-ml. tared Erlenmeyer flask, add 200 ml. of *benzene,* and reflux for 1 hour, heating in a water bath, without attempting to secure complete extraction. Evaporate the solvent, dry 2 hours at 75 to 80°, and weigh.

Titration. Dissolve the fat in 40 ml. of *1 + 1 ethanol-ether solvent* and titrate with *standard 0.01 N sodium hydroxide solution,* using *phenolphthalein* (or for dark-colored fat *thymolphthalein*) *indicator.*

CALCULATION. Express the result as *acidity number,* which is the milliliters of 0.01 *N* potassium hydroxide solution required to neutralize the free fatty acids of 1 g. of fat.

EXAMPLES. Acidity number of freshly milled flour 15 to 20, very old flour as high as 130 to 140.

Zeleny and Coleman Method.[46] The method is designed for all cereal grains as well as flour; a rapid method for corn is described under Part II, A1.

REAGENT. *Benzene-Ethanol-Indicator Solvent.* Add 1 liter of benzene to 1 liter of ethanol containing 0.4 g. of phenolphthalein in solution.

PROCESS. *Sample.* Obtain a sample of 100 g. of corn or 50 g. of other cereals by quartering or by the use of a mechanical sampler and grind so that at least 90% passes a 40-mesh sieve. If too moist for grinding, dry at 100° only long enough to remove excess moisture. Flour needs no further grinding.

Extraction. Weigh 10 (±0.01) g. of the sample immediately after grinding into a continuous extractor and extract for about 16 hours with *naphtha.* Completely evaporate the naphtha from the extract on the steam bath and dissolve the residue in 50 ml. of *benzene-ethanol-indicator solvent.*

Titration. Carry the titration with *carbonate-free 0.0178 N potassium hydroxide solution* to a distinct pink or, if the solution is yellow, to an orange-pink. If an emulsion forms, dispel it by adding a second 50-ml. portion of the *benzene-ethanol-indicator solvent.*

Color Standard. Use, for comparison in

fixing the end-point, a solution prepared as follows. To 50 ml. of water, add dropwise *0.5% potassium dichromate solution* to match the color of the extract before titration, then add 2.5 ml. of *0.01% potassium permanganate solution.*

Blank. Make a blank titration on 50 ml. of the *benzene-ethanol-indicator solvent.*

CALCULATION. Subtract from the number of milliliters of standard potassium hydroxide solution used in the titration of the flour extract the number used for the blank (or blanks) and multiply by 10. The product is the *fat acidity* in terms of number of milligrams of potassium hydroxide required to neutralize the free fatty acids from 100 g. of the sample. Calculate the results to the dry basis.

LIPOIDS

As introduced by Overton,[47] the term lipoids includes all substances soluble in ethanol, as well as fat solvents. Maclean,[48] however, recommends dropping the term because of the confusion in usage. There appears to be no advantage in including fats with the phosphatides in the extract, the chief purpose of which is a step in the determination of the egg content of alimentary pastes under which head the subject is further discussed.

Official A.O.A.C. Method.[49] PROCESS. A. LIPOIDS. *Ethanol-Ether Extraction.* Weigh 5 g. of the sample into a 200-ml. nursing bottle, add 15 ml. of *70% ethanol*, mix by gentle rotation, and digest for 15 minutes in a water bath at 75 to 80° with frequent rotation. Add 27 ml. of *ethanol*, stopper, and shake well for 2 minutes, then cool, add 45 ml. of *ether*, and shake again for 5 minutes, thus preventing lumping. Centrifuge cautiously, avoiding packing, and decant into a 250-ml. beaker containing bits of *porcelain* or *glass*, rinsing with *ether*.

Ether Extraction. Shake the residue for 2 minutes with 20 ml. of *ether*, centrifuge, and decant into the beaker containing the first extract. Repeat the shaking, centrifuging, and decanting twice, then evaporate the joint extract to dryness on the steam bath and dry in an oven at 100° for 5 minutes to remove the moisture on the sides.

Chloroform Extraction. Dissolve the dry extract in about 15 ml. of *chloroform* and filter through a pledget of cotton into a tared platinum dish, washing with *chloroform* and using a rod to break up lumps. Evaporate the filtrate and washings and dry at 100° to constant weight, which requires from 75 to 90 minutes.

B. LIPOID PHOSPHORIC ACID. Redissolve in *chloroform* the extract obtained by the preceding method, add 5 to 10 ml. of *4% ethanolic potassium hydroxide solution*, evaporate, and char at a faint red heat. Add to the charred residue *1 + 9 nitric acid* to slight acid reaction, warm on the steam bath, filter, wash with hot water, and determine phosphoric acid in the solution.

ACIDITY

Although it has been abundantly demonstrated that the solvents for flour acidity are selective, full advantage of this selectivity has not been taken by the A.O.A.C. The solubility of acid-reacting substances by water is chiefly indicative of grade, whereas the solubility of fatty acids by alcohol is indicative of spoilage. At present the Association has no method for acidity that is a test of grade, that is, degree of freedom from bran coats and germ, but it retains in the chapter on stock foods and grain a method that is primarily a grade test for flour although of doubtful value in the examination of cattle foods, which are admittedly rich in bran and germ tissues.

The recently adopted Zeleny naphtha extraction method for the determination of fat acidity of flour (or grain) is believed to fill the need for a spoilage test more satisfactorily

than similar earlier tests, but the need of a flour grade test based on acidity is now unfilled.

The value of titratable water-soluble acidity of flour as an index of grade was first brought to notice by Snyder [50] who earlier first proposed ash for the same purpose.[51] Neither the acidity, as determined in the water extract, nor the ash represents a single substance nor all of some of the constituent substances; nevertheless like much-abused crude fiber, it serves a definite purpose. Swanson [52] showed that soluble phosphates and amino acids are among the substances titrated, although the results are expressed as percentage of lactic acid.

The Mitchell method, long an A.O.A.C. tentative method, but recently abandoned, as found by Winton and Hanson [53] clearly differentiates patent flour from clear, provided a uniform temperature is adopted. It was never designed as a test for spoilage. Markley and Bailey [54] showed that the water-soluble acidity is more closely correlated with the ash content than the alcohol-soluble acidity.

Schindler [55] adopted 85% ethanol as the solvent, since his efforts were directed toward the detection of spoilage of corn (maize) meal. The so-called Greek method is practically the Schindler method applied to flour.

The subject is further treated by Eifield and Bailey,[56] Collatz,[57] Brooke,[58] Schulerud,[59] and more recently by Zeleny and Coleman.[60]

Mitchell Water Extraction Volumetric Method.[61] The method, as given herewith, differs from the original method only in that the weight of flour and the normality of the standard alkali are so adjusted as to simplify calculation.

REAGENT. *Carbon Dioxide-Free Water.* Distilled water often contains free carbon dioxide. Remove by boiling in a Jena flask until just neutral as shown by the failure of a single drop of the standard alkali to produce a pink color in 200 ml. with phenolphthalein as indicator.

PROCESS. Digest at 40° for 1 hour with occasional shaking 18 g. of the flour with 200 ml. of *carbon dioxide-free water* in a 500-ml. volumetric flask, avoiding evaporation. Filter through a fluted paper, returning the first portions to the filter to insure a clear filtrate. Titrate 100 ml. of the filtrate with *0.05 N sodium hydroxide solution,* using *phenolphthalein indicator.*

CALCULATION. Multiply the number of milliliters of 0.05 N alkali by 0.05 to obtain the per cent of acidity calculated as lactic acid.

EXAMPLES. Hard wheat: patent 0.126, clear 0.306%. Soft wheat: patent 0.131, clear 0.216%. All the samples were lightly bleached with nitrogen peroxide, but this had no appreciable effect on the results.

REDUCING AND NON-REDUCING SUGARS

Sandstedt Ferricyanide Volumetric Method.[62] This method differs from that for the determination of diastatic activity given in a subsequent chapter in that the solution is incubated prior to the titration of the reducing sugars, thus hydrolyzing an amount of non-reducing sugar equivalent to the diastatic activity.

The basic principle of the sugar titration is that of the Gentele Method (Part I, C6*a*) which has been variously modified.

REAGENTS. *Acid Buffer Solution.* Dilute a mixture of 3 ml. of glacial acetic acid, 4.1 g. of anhydrous sodium acetate, and 4.5 ml. of H_2SO_4 to 1 liter.

Sodium Tungstate Solution, 12%. Dissolve 12 g. of $Na_2WO_4 \cdot 2H_2O$ in water and dilute to 100 ml.

Ferricyanide Solution, 0.1 N, alkaline. Dissolve 33 g. of dry $K_3Fe(CN)_6$ and 44 g. of anhydrous Na_2CO_3 in water and dilute to 1 liter. Standardize by adding 10 ml. of the solution to 25 ml. of acetic acid-salt solution

MALTOSE AND SUCROSE FROM FERRICYANIDE REDUCED (SANDSTEDT)*

0.1 N Ferri-cyanide	Maltose per 10 g. of Flour	Sucrose per 10 g. of Flour	0.1 N Ferri-cyanide	Maltose per 10 g. of Flour	Sucrose per 10 g. of Flour	0.1 N Ferri-cyanide	Maltose per 10 g. of Flour	Sucrose per 10 g. of Flour
ml.	mg.	mg.	ml.	mg.	mg.	ml.	mg.	mg.
0.10	5	5	3.10	156	148	6.10	341	290
0.20	10	10	3.20	161	152	6.20	347	294
0.30	15	15	3.30	166	157	6.30	353	299
0.40	20	19	3.40	171	161	6.40	360	304
0.50	25	24	3.50	176	166	6.50	367	309
0.60	31	29	3.60	182	171	6.60	373	313
0.70	36	34	3.70	188	176	6.70	379	318
0.80	41	38	3.80	195	181	6.80	385	323
0.90	46	43	3.90	201	185	6.90	392	328
1.00	51	48	4.00	207	190	7.00	398	333
1.10	56	52	4.10	213	195	7.10	406	337
1.20	60	57	4.20	218	200	7.20	412	342
1.30	65	62	4.30	225	204	7.30	418	347
1.40	71	67	4.40	231	209	7.40	425	352
1.50	76	71	4.50	237	214	7.50	431	357
1.60	80	76	4.60	244	218	7.60	438	362
1.70	85	81	4.70	251	223	7.70	445	367
1.80	90	86	4.80	257	228	7.80	451	372
1.90	96	91	4.90	264	233	7.90	458	377
2.00	101	95	5.00	270	238	8.00	465	382
2.10	106	100	5.10	276	242	8.10	472	387
2.20	111	104	5.20	282	247	8.20	478	392
2.30	116	109	5.30	288	251	8.30	485	397
2.40	121	114	5.40	295	256	8.40	492	402
2.50	126	119	5.50	302	261	8.50	499	407
2.60	130	123	5.60	308	266	8.60	505	...
2.70	135	128	5.70	315	270	8.70	512	...
2.80	140	133	5.80	322	275	8.80	519	...
2.90	145	138	5.90	328	280			
3.00	151	143	6.00	334	285			

* These values are arbitrarily given for 10 g. of flour, although the determination is made on only 0.5 g. of flour.

and 1 ml. of starch-iodide solution and titrating with 0.1 N thiosulfate.

Acid-Salt Mixture. Dilute a mixture of 200 ml. of glacial acetic acid, 70 g. of KCl, and 40 g. of $ZnCl_4 \cdot 7H_2O$ to 1 liter.

Starch-Iodide Solution. Dissolve 2 g. of soluble starch in a small quantity of cold water and pour slowly into boiling water with stirring. Cool thoroughly to avoid darkening, add 50 g. of KI, make up to 100 ml. with water, then add 1 drop of saturated NaOH solution.

Standard Thiosulfate Solution, 0.1 N. Dissolve 24.82 g. of $Na_2S_2O_3 \cdot 5H_2O$ and 3.8 g. of $Na_2B_4O_7 \cdot 10H_2O$ in water and dilute to 1 liter. Make a blank determination daily as follows. To a mixture (used in place of 5 ml. of flour extract) of 5 ml. of ethanol, 50 ml. of the acid buffer solution, and 2 ml. of sodium tungstate solution, add 10 ml. of ferricyanide solution and proceed as for reducing sugars below. Ten milliliters of thiosulfate solution should decolorize the blue color. If the titration falls within 30 (± 5) in subsequent sugar calculations, use equivalent to 10 ml. of ferricyanide instead of 10 in subtraction.

PROCESS. *Extraction.* Weigh 5.675 g. of flour into a 100- or 125-ml. Erlenmeyer flask, incline the flask so that the flour is in one place, moisten with 5 ml. of *ethanol*, and add 50 ml. of *acid buffer solution* in such a manner as not to come in contact with the flour until the latter is all added, then shake so as to form a flour suspension.

Clarification. Add at once 2 ml. of *sodium tungstate solution*, mix thoroughly, and filter without delay through a Whatman No. 4 paper, discarding the first 8 to 10 drops.

Ferricyanide Treatment. Pipet into each of two 1 x 8 in. test tubes (*A* and *B*) 5 ml. of the clear filtrate. Pipet directly into tube *A* (for determination of reducing sugars) 10 ml. of *ferricyanide solution*. Heat tube *B* (for determination of non-reducing sugars) by immersion for 15 minutes in vigorously boiling water, cool under the tap, and then add 10 ml. of *ferricyanide solution*. Treat both tubes thereafter in exactly the same manner as follows.

Immerse without delay in vigorously boiling water so that the containing liquid is 3 to 4 cm. below the surface of the water in the bath. Keep in the bath exactly 20 minutes, cool under the tap, and pour into a 100- or 125-ml. Erlenmeyer flask, rinsing the test tube with 25 ml. of *acid-salt mixture*.

Titration. Add 1 ml. of the *starch-iodide solution*, then deliver *standard 0.1 N thiosulfate solution* from a 10-ml. micro buret until the blue color is discharged.

CALCULATION. *Reducing Sugars.* Subtract the number of milliliters of thiosulfate solution used in the titration of *A* from 10. Correct for a slight figure obtained in a blank ferricyanide–thiosulfate titration by subtracting it from the thiosulfate equivalent of the ferricyanide, then find the milligrams of maltose equivalent to the milliliters of ferricyanide reduced in the table

Non-Reducing Sugars. From the number of milliliters of ferricyanide solution reduced after hydrolysis (*E*), subtract the number of milliliters reduced by the maltose of the flour (*A*). The difference represents the milliliters of non-reducing sugars. Find the corresponding milligrams of sucrose in the table above.

STARCH

See Starch, Part I, C6*a*.

TOTAL COLOR

The color of flour is due partly to (1) *endosperm pigments*, largely carotenoids, which together with the fat are dissolved by gasoline, naphtha (petroleum ether), or other petroleum fat solvents, as well as by ether, chloroform, carbon tetrachloride, and carbon bisulfide, and partly to (2) *bran pigments*. The content of endosperm pigment does not

influence baking qualities or flavor. On the other hand, a high content of carotenoids with vitamin A potency is nutritionally highly desirable. The content of bran color is not a measure of bran content and milling grade, since a high content of white wheat bran may contribute no more color than a low content of red wheat bran.

Pekar Test. This test serves to show the total color as compared with standard samples.

APPARATUS. *Glass Plate*, 12 to 15 cm. long and about half as broad.

Flour Slicker, or spatula, about 12.5 cm. broad with a truncated end and a handle of the same thickness as the blade.

PROCESS. By means of the slicker, place a suitable quantity of the sample on the plate and form a straight edge on one side. Treat in like manner a portion of the standard flour and move so that the straight edges of sample and standard are adjacent and compare the color. Flour millers are expert in performing this test.

TOTAL COLOR; CAROTENE

Munsey Ether-Naphtha-Methanol Neutral Wedge Photometric Method.[63] APPARATUS. *Neutral Wedge Photometer*, described by Clifford and Wichmann [64] and modified by the designer by the substitution of a permanent glass wedge for the gelatin wedge.

PROCESS. *Saponification.* Reflux 20 (flour, semolina, or macaroni), 10 (egg noodles), or 2 (egg yolk) g. of the finely ground sample in a 125-ml. Erlenmeyer flask with 50 ml. of *saturated ethanolic potassium hydroxide solution* (10% in ethanol) for 30 minutes on a steam bath. Rotate occasionally but avoid distribution of the material over the sides. Cool to room temperature, filter, using a Büchner-type fritted glass filter (11G3), rinse with a little *ethanol* from a wash bottle, then without suction rinse with 25 ml. of *ether* with stirring on the filter, repeating twice.

Ether Extraction. Transfer the filtrate to a 250-ml. glass-stoppered separatory funnel, rinsing with 25 ml. of *ether*, ignoring resinous matter. Add 175 ml. of cold tap water, carefully invert, and rotate several times. When the separation is complete, draw off the lower aqueous ethanol layer and extract this again with 25 ml. of *ether*. Reject the lower layer and add the ether solution to the original ether solution. Wash the combined solutions by pouring 50 ml. of tap water through it. When the layers separate, draw off and reject the aqueous layer.

Naphtha Treatment. To the ether solution, add 50 ml. of *naphtha* (b.p. 30 to 60°) and wash 5 times with 50 ml. of tap water by inverting and rotating gently. Reject all aqueous layers and disregard slight emulsions. Transfer the ether-naphtha mixture to a 250-ml. distillation flask and heat in a beaker of water at 45 to 50°. Stopper, connect the side arm with a vacuum and concentrate to about 5 ml. to remove ether. Filter through paper two-thirds filled with *anhydrous sodium sulfate* into a 100-ml. volumetric flask and dilute to the mark with *naphtha*, also passed in portions through the sulfate.

(If a determination of total color is desired at this point, pipet a portion of the ether-naphtha solution into a 4-in. absorption cell, without loss, and read the color value in the neutral wedge photometer, using filter No. 44. Then return the solution to the flask.)

Xanthophyl Removal with Methanol. Transfer the solution to a 125-ml. separatory funnel, rinsing with a little *naphtha*. Add 15 ml. of *92% methanol* (92 volumes of absolute methanol plus 8 volumes of water), shake for 10 minutes in a mechanical shaker, or 2 to 3 minutes by hand, and allow the layers to separate in an upright position. Decant the lower layer containing the xanthophyl and repeat the extraction 5 times or until the aqueous-methanol layer is colorless as viewed over a white background. Noodles with

high egg content may require 10 extractions.

Methanol Removal. Wash the naphtha solution with 25 ml. of water by inverting several times, decant, reject the aqueous layer, repeating with two more portions. Transfer the naphtha layer to a 250-ml. distillation flask, place in water at 45 to 50°, and concentrate as before by suction until the volume is reduced to 10 to 15 ml. Filter as before through *anhydrous sodium sulfate* into a 25- to 50-ml. volumetric flask, washing the filter with *naphtha* in diluting to volume.

Color Reading. Mix the naphtha solution and read the color value in a 4-in. cell in the photometer, using filter No. 44.

Calculation. From a standard curve obtain the carotene concentration. Divide by 4 or 2, depending on the volume, to obtain the concentration in 100 ml.

The total color value, calculated as carotene, may be obtained from the same curve, as both xanthophyl and carotene show nearly identical absorption with No. 44 filter. Since the dilution for total color is 100 ml., to convert to gammas per gram in the sample multiply by 5 or 10 for 20 and 10 charges respectively.

Calibration Curve. Dissolve 100 mg. of a natural mixture of *α- and β- carotene* in 5 to 6 ml. of *carbon disulfide*, add 35 to 40 ml. of *ethanol*, cool in the refrigerator for about 1 hour to insure maximum crystallization, and filter on a hardened paper. Dissolve the crystals in 5 to 6 ml. of *carbon disulfide*, add 40 ml. of *naphtha*, cool in the refrigerator as before, filter on a hardened paper, and dry the crystals in a vacuum desiccator for 1 hour.

Weigh exactly 20 ml. of the purified crystals and wash with 20 ml. of *anhydrous ether* into a 1-liter volumetric glass-stoppered flask; dilute to the mark with *naphtha* as soon as the carotene dissolves. Prepare from this stock solution a series of solutions by diluting with *naphtha* portions of 1.25 to 10.0 ml. by 1.25 increments to 250 ml. in volumetric flasks. These represent 0.10 to 0.80 γ/ml. in 0.10-g. increment. Make 10 photometric readings on each solution in a 4-in. cell, using a No. 44 light filter in the photometer eyepiece. Also take the reading of the solvent, which should be exactly zero on the scale.

Plot the average of each reading against the concentration (gammas per milliliter). The line of best fit is obtained by the method of least squares, thus: Let x = the scale reading and y = the concentration in gammas per milliliter. Substituting in $y = a + bx$ the values for a and b as follows:

and

$$a = M_y - bM_x$$

Within the range of the 8 standard solutions the curve is exactly in accordance with Beer's law. Schertz has shown that Beer's law holds up to 3 γ/ml.

Note. In connection with this method, involving one of the most important determinations in food chemistry, Munsey explains the laws of Beer[65] and Lambert[66] which play important parts in colorimetry, photometry, and spectrophotometry in general. By combinations of these laws, the following equations are obtained.

and

in which C is the molar concentration (in the present instances gammas per milliliter), E is the molar extinction coefficient, L is the inside cell diameter or length, and T is the transmittancy, which is the ratio of the transmission through solution and solvent, and $-\log T$ is the absorbancy.

Endosperm Color

(Color Values of Fat-Solvent Extracts)

Nature of Pigments. Markley and Bailey[67] state that the following pigments are present in the gasoline extract of flour: carotene, xanthophyl, reddish orange pigment, tricin, unidentified yellow pigments, and flavones. Zechmeister and Cholnoky[68] found that the endosperm color of wheat and flour is due chiefly to xanthophyl and its esters with only a small amount of α- and β-carotene, chlorophyl and its various decomposition products, and flavones. The specific transmission of xanthophyl and that of carotene are nearly the same. Zechmeister, Cholnoky, and Neumann,[69] by ether extraction and chromatographic analysis, obtained in unbleached flour 0.25 γ/g. of xanthophyl, but only 0.01 γ/g. of carotene.

Influence of Aging and Bleaching. The carotenoids of flour disappear slowly during aging, owing, it is claimed, to the action of nitrogen peroxide or other nitrogenous compounds present in minute amount in the air. The fact that flour takes on a minute amount of a nitrous acid reacting substance after aging for several months, as shown by unpublished tests by Winton and collaborators at the U. S. Food Inspection Laboratory at Chicago, and later results reported by Monier-Williams,[70] is in accord with this claim.

The demand for white flour appears to the nutritionist, who thinks of vitamins, and the epicure, who stresses flavor, as unwarranted. After flour has been artificially bleached, it is comparable with butter colored with a non-vitamin coal-tar color.

During the early years of the U. S. Food and Drugs Act, the government, in the lower court, won a celebrated case involving bleaching, but the verdict was reversed in the higher courts because of a flaw in the charge to the jury and the case was ordered retried. So far as the writers know, no retrial has taken place and bleaching is allowed if declared on the label.

The bleaching process, which the federal authorities combatted, depended on the action of nitrogen peroxide formed by a flaming discharge of electricity in a closed chamber whereby nitrous acid, a poisonous substance, free or combined, was shown to be left in the flour. Other processes (pp. 453, 454) are now extensively used. The standard for flour (p. 434) gives oxides of nitrogen, chlorine, nitrosyl chloride, nitrogen trichloride, and benzoyl peroxide (1 part with 6 of potassium aluminum sulfate and calcium sulfate or magnesium carbonate) as optional ingredients requiring a declaration "bleached," but does not require naming the ingredients.

Classification of Methods. In the *A.A.C.C. Cereal Laboratory Methods*, the grouping of methods is: (1) spectrophotometric, (2) colorimetric, and (3) gasoline color value. In reality there are only two groups: (1) spectrophotometric, that is, measurement of transmittancy, and (2) comparison with potassium chromate directly or indirectly in various ways. The kind of solvent is incidental. Other petroleum solvents may be substituted for gasoline which, like naphtha (petroleum ether), is an indefinite generi- term. Automobile gasoline in 1908 was colorc less and of low boiling point; today, even if no pigment is added, it is commonly yellowish and needs redistillation.

I. Spectrophotometric Method. **Ferrari Spectrophotometric Method.**[71] The method as proposed by Ferrari (General Mills, Minneapolis) has been modified as to solvents by Binnington, Sibbitt, and Geddes[72] and by Binnington and Geddes[73] and adopted by the A.A.C.C.[74] as follows.

Apparatus. *Spectrophotometer*, or a polarization photometer, together with a mercury vapor light, with light filter for isolating wave length 435.8 mμ, and provision for securing two beams of equal intensity.

Absorption Cells, 5 and 10 cm.

Centrifuge, with 100-ml. lipless tubes.

REAGENTS. *Naphtha-Ethanol Solvent.* Mix 93 volumes of naphtha with 7 volumes of absolute ethanol. If not colorless, redistil in glass.

Water-Saturated Butanol, 1.0 N, is an alternate solvent.

PROCESS. *Extraction.* A. *With Naphtha-Ethanol.* Shake vigorously with a rotary motion 20($\pm$0.05) g. of flour in a glass-stoppered bottle with 100 ml. of the *solvent* until the flour is dispersed, then shake at intervals for 1 hour and allow to stand in the dark overnight. Reshake, allow to settle partially, decant into a centrifuge tube, cover with a rubber cap, and centrifuge at 2200 r.p.m. for 20 minutes or until clear. Allow to come to rest very slowly and siphon the clear solution through a capillary siphon.

B. With Water-Saturated 1.0 N Butanol. Shake 20 g. of the sample with 100 ml. of the *solvent*, allow to stand 15 minutes in the dark, reshake, and filter through a 15-cm. Whatman No. 1 filter paper into a small Erlenmeyer flask.

Transmittancy Determination. Fill one absorption cell with the extract and a duplicate cell with the solvent. Make at least ten readings of transmittancy at $\lambda = 435.8$ mμ, shifting the extract and solvent to the alternate beams after the fifth reading.

CALCULATION. Obtain the parts of the pigment expressed as carotene in gammas per gram (C) of flour by the following formula:

$$C = 50 \times \frac{-\log_{10} T}{bK}$$

in which b is the cell length in centimeters, T is transmission of solution divided by transmission of solvent, K is the specific transmission index of carotene at $\lambda = 435.8$ mμ in the solvent (for naphtha-ethanol, $K =$ 1.9165, for water-saturated 1 N butanol, $K = 1.6632$). The calculation is obviated by consulting the two-page table given in the *A.A.C.C. Cereal Laboratory Methods.*

II. Colorimetric Methods Employing Potassium Chromate Standard. **Gasoline Color Value Method.**[75] The method, devised as an aid in the detection of bleaching, has come to be used as a measure of the fat-soluble color, regardless of its composition or the circumstances affecting its intensity.

Incidentally, it may be stated that organic pigments in certain other foods are now measured in terms of various inorganic standards. Among the suggested organic standards are alizarin in chloroform, naphthol yellow and orange G in water, and azobenzene in ethanol.

APPARATUS. *Colorimeter*, of any type.

REAGENTS. *Colorless Gasoline.* The gasoline sold at filling stations, even when not artificially colored, is not water white. It must be distilled if used for determining the color value. Naphtha (petroleum ether; canadol) is a satisfactory, but more expensive, substitute. In all cases, a blank determination should give a value of zero.

Standard Potassium Chromate Solution, 0.005%. Prepare a stock solution by dissolving 5 g. of crystallized K_2CrO_4 in 1 liter of water. For use, dilute 10 ml. of the stock solution up to 1 liter; this solution has a gasoline value of 1.0.

PROCESS. *Solution.* Weigh 20 g. of the sample into a wide-mouth glass-stoppered, 4-ounce (120-ml.) bottle. Add 100 ml. of *colorless gasoline*, measured by a pipet, stopper tightly, and shake vigorously for 5 minutes. After allowing to stand 16 hours (overnight), shake again until the flour has been loosened from the bottom of the bottle, thus insuring thorough mixing. Filter without delay on a dry 11-cm. paper, which in advance has been fitted to the funnel with water and thoroughly dried, keeping the funnel closed with a watch-glass and collecting the filtrate in a flask.

A clear solution may be secured by allowing a portion of the flour to pass over onto

the filter and returning the first portions of the filtrate to the funnel.

Color Comparison. Compare the color of the filtrate with that of the *0.005% potassium chromate solution* which corresponds to a gasoline color value of 1.0. If a colorimeter is used, adjust the gasoline solution in one tube to a definite mark, then raise or lower the other tube containing the standard potassium chromate solution or the plunger until the shades in both tubes match.

CALCULATION. Divide the reading of the potassium chromate solution by the reading of the gasoline solution, thus obtaining the gasoline color value.

EXAMPLES. The following table[76] illustrates the application of the method.

Description of Samples	Minnesota Hard Spring		Nebraska Hard Winter		Michigan Soft Winter		Missouri Soft Winter	
	78% patent	22% clear	80% patent	20% clear	80% patent	20% clear	40% patent	60% clear
Unbleached								
New (February)	2.00	2.00	2.63	2.50	1.43	1.61	1.47	1.60
Aged 10 weeks	1.78	1.82	2.12	2.17	1.22	1.49	1.22	1.33
Aged 20 weeks	1.20	1.34	1.36	1.68	0.80	1.20	0.68	0.88
Aged 30 weeks	0.72	0.88	0.70	0.82	0.56	0.72	0.48	0.52
Bleached *								
New (February)	0.60	0.66	0.80	0.80	0.40	0.50	0.32	0.40
Aged 10 weeks	0.44	0.54	0.46	0.48	0.20	0.38	0.22	0.26
Aged 20 weeks	0.30	0.50	0.34	0.40	0.20	0.36	0.18	0.24
Aged 30 weeks	0.30	0.50	0.24	0.36	0.18	0.40	0.14	0.16

* All samples were bleached with nitrogen peroxide so as to contain about 2 γ of nitrite nitrogen; after 30 weeks, only a trace remained.

I. Jørgensen Buffer Modification.[77] This modification differs from the original method in that the 0.005% potassium chromate solution is buffered at *p*H 5.59.

REAGENT. Dissolve 0.05 g. of K_2CrO_4 in 1 liter of Sørensen buffer solution made by mixing 950 ml. of $M/15$ KH_2PO_4 solution (9.078 g. per liter) with 50 ml. of $M/15$ $Na_2HPO_4 \cdot 2H_2O$ solution (11.876 g. per liter). This solution, like that of the original method, has a gasoline color value of 1.0 and is claimed to be more stable than a water solution which may contain carbon dioxide or alkali from the glass.

PROCESS and CALCULATION. As in the original method.

II. Kent-Jones and Herd Modification.[78] Instead of potassium chromate alone, a mixture of *potassium chromate* and *cobalt nitrate* is used.

Geddes, Binnington, and Whitside Standard Series Naphtha-Ethanol Method.[79] Although the comparisons are made against solutions of potassium chromate, the results are calculated in terms of carotene. The method, as first proposed, was modified by Binnington and Geddes (Winnepeg) as follows.

APPARATUS. *Nessler Tubes*, of clear glass, polished plane-parallel bottoms, length 150

mm., width 24 mm., wall thickness 1.5 mm., with graduation marks at 40 and 80 mm. inside measurement.

Color Comparator, such as is used in water analysis.

Lamps, quartz mercury (Alpine sun lamp or 250-watt mercury vapor lamp similar to General Electric Company Type H-2).

REAGENTS. *Solvents* as in the Ferrari spectrophotometric method above.

Potassium Chromate Solution, 0.5%. From this stock solution prepare a series corresponding to even increments of carotene based on a flour-solvent ratio of 1 : 5 as given in the following table.

Carotene Value	0.5% K_2CrO_4 per Liter	
	Naphtha-ethanol extract	Butanol extract
γ/g.	ml.	ml.
0.6	2.73	2.33
0.8	3.62	3.10
1.0	4.51	3.88
1.2	5.39	4.65
1.4	6.28	5.43
1.6	7.16	6.20
1.8	8.05	6.98
2.0	8.94	7.75
2.2	9.82	8.53
2.4	10.71	9.30
2.6	11.59	10.08
2.8	12.48	10.85
3.0	13.37	11.63
3.2	14.25	12.43
3.4	15.14	13.18
3.6	16.02	13.95
3.8	16.91	14.73
4.0	17.80	15.50

PROCESS. *Extraction*. Prepare flour extracts as in the Ferrari spectrophotometric method above and make the observations in a dark room in the usual manner.

Color Comparison and Calculation. Compare with the *standard* and report the result as found in the table.

A.A.C.C. Colorimetric Method. The colorimetric procedure of the A.A.C.C. differs from the other colorimetric procedures in this section in that the solvents are the same as for the spectrophotometric method and the standard solution has a carotene value of 4 γ/g. in accordance with the Geddes, Binnington, and Whitside method above. The calculation is the same as for the gasoline color value, except that the standard reading divided by the flour extract reading is multiplied by 4 to obtain the result in terms of carotene.

BRAN COLOR

Müller Ethanol-Ammonia Colorimetric Method.[80] APPARATUS. *Colorimeter* or *Pulfrich Photometer*.

REAGENT. *Standard Potassium Dichromate Solutions*, as per table.

PROCESS. *Ether Extraction*. Weigh 5 g. of the sample into a sintered glass crucible (Schott No. 1G3), attach to an exhaustion flask, and extract with 3 to 5 portions of 10 ml. each of *ether*, with stirring, until all the carotenoids have been removed.

Ethanol Extraction. Remove the ether by suction and transfer the extracted residue by means of a brush and funnel to a 20-ml. centrifuge tube. Add 10 ml. of *75% ethanol*, mix by stirring, and finally by shaking vigorously for 1 minute. Centrifuge and decant the clear liquid into a dry test tube.

Color Formation. Add 1 ml. of *25% ammonium hydroxide* and 0.2 g. of *Kieselguhr*, shake, and filter into a colorimeter tube or photometer cell.

Color Comparison. Match against one of a series of 20 solutions containing 0.030 to 2.082% of *potassium dichromate* or determine the color value in a Pulfrich photometer. Express results in terms of ethanol-ammonia

color value (bran coloring matter) in accordance with the table herewith.

Ethanol-Ammonia Color Value	Potassium Dichromate %	Photometer Reading
1	0.030	16
2	0.038	18
3	0.047	21
4	0.059	25
5	0.073	30
6	0.092	35
7	0.115	40
8	0.143	46
9	0.179	52
10	0.224	59
11	0.279	66
12	0.349	72
13	0.437	77
14	0.546	81
15	0.682	84
16	0.853	86
17	1.066	88
18	1.333	89.5
19	1.666	91
20	2.082	92

Thiamin; Riboflavin

See Part I, C10, and Part II, A1.

Nicotinic Acid

Swaminathan Aniline-Cyanogen Bromide Colorimetric Method. See also Part I, C10, and Part II, A3.

I. Melnick, Oser, and Siegel Modification.[81] Suited also for bread.

Apparatus. *Photoelectric Colorimeter.*

Process. *Extraction.* Weigh 2 g. of flour or air-dried bread into a 250-ml. Erlenmeyer flask, add 100 ml. of water, and autoclave the suspension for 30 minutes at 15 pounds pressure, then centrifuge and wash twice with 50 ml. of boiling water. Concentrate the extract and washings *in vacuo* or in an oven at 115° overnight to a volume of 5 ml. or less. Add 5 ml. of *hydrochloric acid*, heat in a boiling water bath, and make up the solution or suspension to 15 ml. in a calibrated test tube.

Hydrolysis. Heat in boiling water for 30 to 40 minutes with stirring, using a few drops of *caprylic alcohol* to control foaming, cool, restore to 15 ml., add 10 ml. of *absolute ethanol*, and transfer to a 125-ml. Erlenmeyer flask. Add 300 mg. of *charcoal*, shake, and filter. Pipet 12.5 ml. of the filtrate into a graduated test tube, add 1 drop of *phenolphthalein*, neutralize to pH 7, and dilute to 15 ml.

Color Formation. To one (a) of three 3-ml. portions of the solution, add 7 ml. of *ethanolic buffer;* to a second (b) add 6 ml. of *cyanogen bromide reagent*, then 1 ml. of *aniline solution* with stirring, and to a third (c) add 0.1 ml. of the *standard nicotinic acid solution* (10 γ) and reagents as to b.

Color Reading. Read after 5 and before 10 minutes in the photoelectric chlorimeter with 420 mμ filter.

Calculation. Subtract reading a from reading b and correlate $c - b$ with the amount of nicotinic acid added.

Convert the galvanometer readings (G) into photometric density (L) by the formula

$$L = 2 - \log G$$

II. Research Corporation Collaborative Modification. The modification is based on the procedures of Melnick and Field and of Dann and Handler, and data supplied by Cannon and Gortner.[82]

Apparatus. *Photoelectric Colorimeter*, with 420 mμ filter.

Reagents. *Cyanogen Bromide Solution*, 4%. Prepare from cyanogen bromide (Eastman), using cold water. Store the crystals in the refrigerator. This replaces the reagent as prepared by Swaminathan.

Aniline Solution, 4%. Dissolve redistilled aniline in absolute ethanol. In a dark bottle, it keeps for months at room temperature.

Buffer Solution. As in the Melnick and Field Modification, except that only 8 ml. of 85% phosphoric acid are used.

Lloyd's Reagent. A form of hydrated aluminum silicate supplied by Eli Lilly & Co., Indianapolis, Ind.

PROCESS. *Hydrolysis.* Weigh into a test tube with marks at 15 and 16.5 ml. a portion of the sample containing 20 to 100γ of nicotinic acid, add 10 ml. of water and 5 ml. of *hydrochloric acid*, then heat in a boiling water bath for 30 to 40 minutes with occasional stirring. Cool to room temperature and adjust to pH 0.5 to 1.0 with about 3 ml. of *18 N sodium hydroxide solution*, using *0.1% aqueous methyl violet solution* as an external indicator on a spot plate.

Adsorption. Add to the solution 2.5 g. of *Lloyd's reagent*, shake vigorously for 1 minute, centrifuge, and discard the supernatant liquid. Disintegrate and wash the residue twice by shaking with 10 ml. of *0.20 N sulfuric acid* and centrifuging.

Elution. To the washed residue, add 12 ml. of *0.5 N sodium hydroxide solution*, shake vigorously for 1 minute, and adjust with water to 16.5 ml.; volume of solution = 15 ml.

Decolorization. Again shake and centrifuge, then drain the eluate as completely as possible into a clean dry test tube containing 0.7 g. of finely powdered *lead nitrate*. Shake for 1 minute, centrifuge, and transfer the solution to another clean dry test tube.

Neutralization. Add 1 drop of *1% ethanolic phenolphthalein solution*, then solid *potassium phosphate* to a slight pink color and 1 to 2 drops of *20% phosphoric acid* from a capillary pipet until a drop on *litmus paper* gives a neutral reaction. Centrifuge, draw the clear supernatant solution through a U-tipped pipet into a special test tube graduated to 15 ml. by 0.1-ml. divisions, note the volume, and dilute to 10 ml.

Color Formation and Color Reading. Proceed as in Modification I, but use reagents as prepared above and observe the following details of technique.

In the color measurement, make two center settings, one for evaluating the residual color, the other for the reaction color. Set the colorimeter to give a galvanometer reading of 100 (zero photometric density) with a solution containing 3 ml. of water and 7 ml. of ethanolic buffer solution. Using the resulting center setting (No. 1 galvanometer reading with the test tube or cuvet containing the pure solution now removed), read the blank test solutions in turn to determine the residual color of each. Then set the colorimeter to give a galvanometer reading of 100 with a solution containing 3 ml. of water, 6 ml. of cyanogen bromide solution, and 1 ml. of aniline solution. Read all subsequent solutions containing reacting nicotinic acid using the new center setting (No. 2). Convert galvanometer readings (G) into photometric density (L) by the formula

$$L = 2 - \log G$$

Some instruments have a dual scale permitting direct reading of L; others are accompanied by a conversion table.

If the cuvet requires between 10 and 20 ml. of solution, dilute the decolorized, neutralized test solutions two-fold with water and use 6-ml. aliquots and double quantities of reagents. If a flat cuvet is used the setting of which is greater than 100 and therefore off the scale, set the galvanometer reading at 50 instead of 100 and multiply the readings by 2.

ASH

The percentage of ash in white flour is an index of milling grade, that is, of the perfection of removal of bran and germ from the endosperm. The figures on ash in the table at the beginning of Part II, A1, clearly illustrate the relationship of ash to grade.

Direct Incineration Gravimetric Method. The reduction of flour in a platinum dish to a

white ash at dull redness in a muffle furnace by the time-honored method is not readily accomplished. The carbonized mass burns slowly and attempts to hasten the burning by increasing the heat are sure to add difficulties since the phosphates fuse about the particles of carbon, effectually preventing contact with the air. Numerous modifications have been proposed to facilitate ashing, but some of these cause loss of ash by too active combustion and others introduce mineral substances, thereby necessitating a blank determination with attendant inaccuracies.

A safe procedure for removing the last traces of carbon is to allow the dish to cool, add sufficient water to cover the bottom of the dish, evaporate, and ignite cautiously. In extreme cases, a few drops of *dilute nitric acid* may be used instead of water without introducing an appreciable error.

Hertwig and Bailey Glycerol-Ethanol Gravimetric Method.[83] By mixing 5 g. of the flour with 10 ml. of 1 + 1 glycerol-ethanol, evaporating, and burning at 575°, these authors (U. S. Depart. Agr., Bureau of Chemistry and Soils) shortened the time of ashing to 1.5 to 2 hours. The A.A.C.C. adopted the method without material change other than reducing the amount of the glycerol-ethanol mixture. Mangels and collaborating members of the A.O.A.C. secured satisfactory results by the method, but found that at the end attention must be given to frothing.

Reagent. *Glycerol-Ethanol Mixture.* Mix equal parts of glycerol and ethanol. A blank determination should yield no appreciable amount of ash.

Process. Weigh 3 to 5 (± 0.01) g. of the sample into a tared platinum dish, which has been heated, cooled, and weighed as in the actual determination. Add 1.5 ml. of the *glycerol-ethanol mixture*, allow to stand for 10 minutes, ignite with a match, and place the dish and contents in a muffle furnace heated at 575°. Heat until the ash is fluffy and gray-white. Cool in a desiccator and weigh quickly as soon as room temperature is attained.

Spalding Magnesium Acetate Gravimetric Method.[84] Brendel[85] proposed ashing 3 g. of flour with 2 ml. of a *N/42 calcium acetate solution* at 1500° F. in an atmosphere of *oxygen*. The method is rapid, but the hygroscopicity of the resulting calcium oxide is a distinct disadvantage. Walters[86] recommends *lanthanum nitrate*, especially for ashing soft wheat flours. Other *nitrates of the rare earth metals* may be used. Kranz[87] omits the oxygen treatment.

I. A.A.C.C. Modification.[88] Reagent. *Ethanolic Magnesium Acetate Solution.* Dissolve 15 g. of $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ in denatured ethanol and dilute to 1 liter. Let stand overnight and filter if not clear.

Process. Weigh 3 (± 0.01) g. of the sample into a flat-bottom platinum, silica, or porcelain dish with a smooth inner surface and thoroughly wet with 3 ml. of the *ethanolic magnesium acetate solution* delivered from a pipet. After allowing to stand 5 minutes, place in a muffle furnace at 850° with the door open, then close and heat 30 to 45 minutes longer until the ash is white or light gray and cool in a desiccator. Loosen the ash by tapping, transfer directly to the balance pan, and weigh.

Calculation. Deduct the weight of the residue obtained in a blank determination (usually 0.0085 to 0.0090 g.) and calculate the ash in the usual manner. A more accurate course is to make parallel determinations by the acetate and the usual method on a similar flour.

II. A.O.A.C. Official Modification.[89] Reagent. *Ethanolic Magnesium Acetate Solution.* Dissolve 4.054 g. of $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ in 50 ml. of water and dilute to 1 liter with ethanol.

Process. Weigh 3 to 5 g. of the sample into a flat-bottom dish and add 5 ml. of the *ethanolic magnesium acetate solution* from a

pipet. (To 1 g. of bran, wheat germ, etc., add 10 ml.) After 1 to 2 minutes, evaporate the ethanol and heat in a muffle furnace at 700°, closing the door after the flame dies down. Burn to a light gray ash, cool in a desiccator, and weigh the dish plus the ash.

Correct for the residue obtained in a blank determination.

Briggs Carbitol–Magnesium Nitrate Gravimetric Method.[90] This method (Howard Testing Laboratory, Minneapolis) may be regarded as a combination of nitric acid treatment with the magnesium acetate method.

Reagent. *Magnesium Nitrate Solution in Carbitol.* Weigh 6.358 g. of $Mg(NO_3)_2 \cdot 6H_2O$ into a 1-liter volumetric flask, dissolve in carbitol (the monoethyl ether of diethylene glycol), and make up to the mark with the solvent.

Process. Weigh 2 g. of patent, 1 g. of clear, or 0.75 g. of low grade flour into a weighed porcelain, silica, or platinum crucible or ignition capsule of 8 ml. capacity. Add 2 ml. of *magnesium nitrate solution* from a pipet, allowing to drain exactly 15 seconds and touching the end to the side of the dish. Heat over a Meker or Fisher burner for about 10 seconds, or until the contents of the crucible begin to burn, then turn off the heat and allow to burn quietly. When the flame dies down, heat again with the burner, or in a muffle furnace for 1 to 2 hours at an orange heat. The ash is white or grayish.

Make a blank determination on 2 ml. of the magnesium nitrate solution and introduce a correction which should closely approximate 2 mg.

Original Ash of Flour

(For Phosphated and Self-Rising Flours)

Gustafson Carbon Tetrachloride Gravimetric Method.[91] Process. Weigh 20 to 25 g. of the sample into a metal centrifuge tube (diameter 5 cm., depth 15 cm.). Add *carbon tetrachloride* to within 2.5 cm. of the top and centrifuge for 5 to 7 minutes at 1600 r.p.m. Allow to come to rest slowly. Skim off in one large tablespoonful as much as possible (about 90%) of the flour forming a compact layer on the surface. Allow the moist flour to dry overnight, weigh, and determine the ash.

Iron in Flour and Bread

Munsey Phenanthroline Colorimetric Method.[92] The method is of particular value in determining the iron due to enriching.

Apparatus. *Neutral Wedge Photometer*, or equivalent instrument.

Reagents. *Phenanthroline Solution.* Dissolve 0.1 g. of orthophenanthroline in about 80 ml. of water, cool, and dilute to 100 ml.

Dipyridyl Solution. Dissolve 0.1 g. of alpha-alpha dipyridyl (Eastman Kodak Co.) in water and dilute to 100 ml. (May be used instead of the phenanthroline solution. Both keep for several weeks if stored in a cool dark place.)

Hydroxylamine Hydrochloride Solution. Dissolve 10 g. of the salt in water and dilute to 100 ml.

Magnesium Nitrate Solution. Dissolve 50 g. of $Mg(NO_3)_2 \cdot 6H_2O$ in water and dilute to 100 ml.

Acetate Buffer Solution. Recrystallize c.p. anhydrous $NaC_2H_3O_2$, if necessary, and dry at 100°. Dissolve 8.3 g. in water, add 12 g. of glacial $C_2H_4O_2$ (redistilled if necessary), and dilute to 100 ml.

Standard Iron Solution. Dissolve 0.1 g. of pure iron wire in 20 ml. of HCl and 50 ml. of water, dilute to 1 liter, then dilute 100 ml. of this solution to 1 liter. If desired, substitute a solution of 3.512 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 2 drops of HCl in 500 ml. of water, further diluted 100-fold. Each milliliter of both solutions = 0.01 g. of Fe.

Reference Curve. Prepare a series of solutions containing 2.0, 5.0, 10.0, 15.0, etc.,

ml. of the iron solution and 2.0 ml. of HCl, diluted to 100 ml. Using 10 ml. of each, proceed as in an actual analysis and plot the concentration against scale readings.

PROCESS. *Sample.* Mix flour thoroughly. Crush bread to about 20-mesh size (about 1 mm.) with a wooden rolling-pin on a wooden board. Note the loss in weight.

Incineration. Reduce to ash 10 g. of the sample with the usual precautions (Part I, C2*f*) until practically carbon-free. If desired, use 0.5 to 1.0 ml. of *magnesium nitrate solution* or *distilled nitric acid* to hasten the burning, but do not add these ash aids to self-rising flour, bread, or products containing salt, if in a platinum dish.

Preparation of Solution. Cool the ash, add 5 ml. of *hydrochloric acid*, and evaporate to dryness on the steam bath. Add to the residue exactly 2.0 ml. of *hydrochloric acid*, heat for 5 minutes on the steam bath under a watch-glass, wash off the watch-glass with water, filter into a 100-ml. volumetric flask, cool, and dilute to volume.

Color Formation. Pipet 10 ml. into a 25-ml. volumetric flask, add 1 ml. of *hydroxylamine hydrochloride solution*, and allow to stand a few minutes. Add 5 ml. of the *acetate buffer solution* and 1 ml. of *phenanthroline solution* or 2 ml. of *dipyridyl solution*, and dilute to volume.

Color Reading. Read the color intensity of the unknown and a blank in a 2-inch cell in the photometer, using a No. 51 filter (±510 wave length).

CALCULATION. Find the weight of iron by reference to the standard curve and calculate the results as milligrams per pound.

Results on the dry basis are also desirable.

CARBON DIOXIDE

(In Self-Rising Flour)

Gravimetric Method. See Part II, K2.

Chittick Gasometric Method.[93] PROCESS. Weigh 17 g. of the sample into the evolution flask, add 15 to 20 glass beads, 4 to 6 mm. in diameter, and 45 ml. of *1 + 5 sulfuric acid*, then proceed with the manipulation as directed for the determination of carbon dioxide in baking powder, except that the evolution flask is agitated vigorously for 3 minutes and allowed to stand 10 minutes to secure equilibrium.

CALCULATION. Subtract the volume of acid used from the total buret reading and correct for temperature and barometric pressure, then divide the corrected reading by 100 to obtain the apparent percentage of carbon dioxide by weight. Correct this apparent percentage by immediately applying the method to a synthetic sample of known composition and like ingredients determined by the same method and in the same apparatus. Divide the weight of carbon dioxide thus found by the weight of carbon dioxide in the sodium bicarbonate used and divide the apparent total carbon dioxide in the official sample by this quotient, thus obtaining the correct percentage of carbon dioxide in the official sample.

BLEACHING RESIDUES

Sulfur fumes are used for bleaching oats and possibly other cereals.

Of the various processes devised to hasten and intensify the bleaching of flour which normally takes place during aging, Munsey[94] mentions the following: (1) *Alsop Process*, patented about 1904, employing nitrogen peroxide (also known as nitrogen dioxide or nitrogen tetroxide, according as the formula NO_2 or N_2O_4 is assumed) produced by a flaming discharge of electricity, (2) *Beta Chlora Process*, with patents from 1907 to 1916, employing chlorine and nitrosyl chloride, $NOCl$, (3) *Agene Process*, employing nitrogen trichloride, NCl_3, patented in 1921, and (4) *Novadelox Process*, employing benzoyl peroxide, $(C_6H_5CO)_2O_2$, and acid calcium phosphate, patented in 1921.

To these should be added the following used in Europe and mentioned by the Danish author Jørgensen:[95] (5) *Golo Process* similar to the Beta Chlora process, (6) *Ozone Process*, and (7) *Hydrogen Peroxide Process;* also the following, mentioned by an anonymous French author [95] and other European authors, (8) *Persulfate Process*, and (9) *Bromine Process*, of which details are not available, that may refer merely to the addition of so-called flour improvers or yeast foods.

Since there is some lack of agreement as to which of the five oxides of nitrogen react in the bleaching of flour, it may be stated that the following do not react directly: (1) nitrous oxide or laughing gas, N_2O, (2) nitric oxide, NO (although it forms nitrogen peroxide on contact with air), (3) nitrogen trioxide or nitrous anhydride, N_2O_3, a green liquid, and (4) nitrogen pentoxide or nitric anhydride, N_2O_5, a solid, melting at 30°.

All bleaching processes cause a decolorization of the flour which may be measured by the gasoline color value (which see).

Nitrous Acid

(Nitrogen Peroxide Bleaching)

The bleaching of flour by nitrogen peroxide, nitrosyl chloride, or other related compounds is detected by qualitative tests and the determination of nitrous acid, free or combined, in the water extract.

Griess-Ilosvay Sulfanilic Acid-Naphthylamine Test. Process. Smooth (slick) a portion of the flour and add a drop of a mixture of equal parts of the *sulfanilic acid* and *naphthylamine reagents* prepared as directed for the Griess quantitative colorimetric method below. There is no material change in color in fresh unbleached flour beyond that which comes with wetting with water, but flour bleached with nitrogen peroxide soon acquires a pink or red color, varying in intensity with the degree of bleaching.

Griess-Ilosvay Sulfanilic Acid-Naphthylamine Colorimetric Method.[96] The method as described below is an adaptation of one long employed in the determination of nitrites in water.

Apparatus. *Nessler Tubes* or *Colorimeter*.

Reagents. *Sulfanilic Acid Solution.* Dissolve 1 g. of sulfanilic acid in a mixture of 60 ml. of glacial acetic acid and 240 ml. of nitrite-free distilled water, with the aid of heat.

α-Naphthylamine Hydrochloride Solution. Dissolve 0.4 g. of the salt in a mixture of 60 ml. of glacial acetic acid and 240 ml. of nitrite-free distilled water with the aid of heat.

Standard Sodium Nitrite Solution. Pure silver nitrite is obtainable at supply houses or may be made as follows. Prepare concentrated solutions in hot water of 8 parts of $NaNO_2$ and 16 parts of $AgNO_3$, mix, and cool. Collect the precipitate on a Büchner funnel, wash with cold water, and dry quickly on a water bath with a minimum of exposure to light. Decomposition takes place on long heating.

Accurately weigh 0.1097 g. of dry $AgNO_2$, dissolve in 20 ml. of hot nitrite-free water, add 0.05 g. of NaCl, shake to cause a flocculation of the AgCl, and dilute to 1 liter in a volumetric flask. Remove with a pipet 10 ml. of the clear supernatant liquid and dilute to 1 liter; 1 ml. = 0.0001 mg. of nitrogen in the form of nitrous acid, free or combined.

Process. *Extraction.* Measure with a pipet into an Erlenmeyer flask 200 ml. of water at 40° and add 20 g. of the flour, then close with a rubber stopper and shake vigorously for 5 minutes. Digest at 40° for 1 hour, shaking every 10 minutes. Filter on a dry nitrite-free pleated paper, returning the first portions of the filtrate to the paper, until a clear solution is obtained.

Aliquots of the same solution may be used for determining both nitrous acid and acidity.

Color Formation. Mix 50 ml. of the filtrate and 50 ml. of *standard sodium nitrite solution* each with an equal volume of water and add 2 ml. each of *sulfanilic acid solution* and *α-naphthylamine hydrochloride solution.* Allow to stand 1 hour, during which time the color develops.

Color Comparison. Compare in Nessler tubes or in a colorimeter.

CALCULATION. Divide the height of the standard solution by that of the solution of the sample, thus obtaining the nitrous acid (free or combined) expressed as gammas per gram of the flour.

CHLORINE IN FAT

(Chlorine Bleaching)

Volhard-Drechsel Silver Nitrate Volumetric Method.[97] During the period when nitrogen peroxide was the common bleaching agent and chlorine was a novelty, one of us [98] employed successfully the following method in conjunction with the determination of the iodine number of the fat (ether extract).

REAGENTS. *Nitric Acid.* Freed from lower oxides by dilution and boiling until colorless.

Standard Silver Nitrate Solution, 0.1 *N*. Standardize against 0.1 *N* NaCl (5.846 g. per liter).

Standard Potassium Thiocyanate Solution, 0.1 *N*. Standardize against 0.1 *N* $AgNO_3$.

Ferric Alum Indicator. Saturated solution of $FeNH_4(SO_4)_2 \cdot 12H_2O$.

PROCESS. *Extraction.* Dry 2.25 g. of the flour in a boiling water oven for 3 hours, extract with *anhydrous ether* in a continuous extractor, transfer the ether solution to a platinum dish, and evaporate the ether.

Ignition. Add to the fat 10 g. of *chlorine-free sodium carbonate,* mix well, and ignite at a dull redness.

Chlorine Precipitation. Dissolve the residue in water slightly acidulated with *nitric acid.* Add an excess of *standard 0.1 N silver nitrate solution,* stir well until the silver chloride coagulates, filter, and wash.

Titration. Add to the filtrate 5 ml. of *ferric alum indicator* and titrate the excess of silver nitrate with *standard 0.1 N potassium thiocyanate solution* to the appearance of a permanent brown color.

CALCULATION. Use the formula: 1 ml. of standard 0.1 *N* silver nitrate solution = 0.00355 g. of chlorine.

NOTE. A procedure differing from the foregoing chiefly in that sodium ethylate is substituted for sodium carbonate, described by Munsey [99] who states that it is essentially the Kent-Jones and Head method, was for a time the tentative A.O.A.C. method for detecting chlorine bleaching, but later gave place to the iodometric method, now official, given below.

Wilkins-Scott Iodide Volumetric Method.[100] The details of extraction and fusion were developed by Scott.[101] The method proper is based on the Christy and Robson method,[102] but biniodate has been replaced by specially purified iodine. In this latter respect, the procedure is like Official Volumetric Method II for the determination of chlorine in plants.[103]

REAGENTS. *Fusion Mixture.* Mix 138 g. of anhydrous K_2CO_3, 106 g. of anhydrous $NaCO_3$, and 75 g. of powdered KNO_3.

Silver Nitrate Solution, 0.3 *N*. Dissolve 48 g. of $AgNO_3$ in water, filter, and dilute to 1 liter; 1 ml. = about 10 mg. of Cl.

Standard Silver Nitrate Solution. Dilute 100 ml. of the 0.3 *N* solution to about 900 ml. and adjust so that 1 ml. = 1 ml. of standard potassium iodide solution.

Copper Sulfate-Potassium Sulfate Mixture. Mix thoroughly 1 part of $CuSO_4 \cdot 5H_2O$ and 16 parts of K_2SO_4.

Standard Potassium Iodide Solution. Dissolve in water 4.6822 g. of KI, dried to constant weight at 105 to 150°, and dilute to 1 liter.

Iodine Solution. Shake a large excess of iodine crystals in a glass-stoppered bottle nearly filled with 35% H_2SO_4 and discard the solution. Repeat and decant into a glass-stoppered bottle. Test 25 ml. of the solution by adding to 25 ml. of dilute H_2SO_4 (3.5 + 100 by volume, boiled 5 to 10 minutes and cooled) and 5 ml. of the starch solution. No blue color should appear after 5 minutes and the color produced by a small amount of standard KI solution should be discharged by an equivalent amount of standard $AgNO_3$ solution. If the solution gives a blue color when tested, compute the quantity of standard $AgNO_3$ solution needed to treat the remainder of the decanted solution from excess of standard $AgNO_3$ solution over standard KI solution observed in test titrations. Add twice that amount and test as before.

Starch Solution. 2.5%. Make to a paste with cold water 2.5 g. of soluble starch, add 25 to 50 ml. of cold water and shake 5 minutes. Centrifuge, decant, and discard the liquid, repeating 3 times, then transfer the residue to a flask, make up to 100 ml. with boiling water, stir, allow to come to a boil, cover, and cool under the tap with occasional shaking.

PROCESS. *Extraction.* Place 500 g. of the sample and 700 ml. of *naphtha* in a large flask and shake at 5-minute intervals for 30 minutes. Filter through a Büchner funnel and evaporate the filtrate on the steam bath to about 10 ml., then filter the extract and rinse with *naphtha* through a pledget of cotton into a 90-ml. nickel crucible containing 20 g. of the *fusion mixture* previously dried for 30 minutes in an oven at 100°, cooled in a desiccator and weighed. Evaporate the naphtha, again dry for 30 minutes, cool, weigh, and obtain the weight of the fat by difference.

Fusion. Spread evenly 5 g. more of the *fusion mixture* over the mixture and burn in a muffle furnace for 1 hour, then cool, add a few milliliters of water, macerate, dry on the steam bath, and burn to a white ash at 600°, repeating if necessary.

Silver Chloride Precipitation. Take up the ash in 25 ml. of hot water and transfer to a 200-ml. tall-form beaker, rinsing with hot water, add *nitric acid* cautiously to slight acid reaction to *litmus paper*, then 25 ml. more of *nitric acid* and 5 ml. of *0.3 N silver nitrate solution.* Boil for 5 minutes, cool, and filter through 9-cm. No. 1 Whatman or other chlorine-free paper, rinsing with *1% nitric acid.*

Digestion. Place the filter and contents in a Kjeldahl flask together with the *copper sulfate-potassium sulfate mixture* and *sulfuric acid* in amount such as required for nitrogen determination and digest in like manner. See Part I, C1, Gunning-Arnold Method.

Titration. Cool the digest, add 175 ml. of water, boil 5 to 10 minutes, cool, and titrate with *standard potassium iodide solution* with the addition of 5 ml. of *starch solution* and 30 ml. of *iodine solution.* If the amount of chlorine is greater than 30 mg., add only 2 ml. of *starch solution* and 10 ml. of *iodine solution* at the start and the remainder when near the acid point, after agitating to coagulate the precipitate.

CALCULATION. Use the formula: 1 ml. of standard iodide solution = 1 mg. of chlorine. Report as milligrams of chlorine per gram of fat.

BENZOIC ACID

(Benzoyl Peroxide Bleaching)

Rothenfusser Benzidine Sulfate Test.[104] The test was used in Germany for detecting Novadelox bleaching.

REAGENT. *Benzidine Sulfate.* Rub up in a mortar 1 g. of the salt (*p*-diaminophenylmonosulfo acid) with ethanol, wash into a flask, make up to about 100 ml. with ethanol, and reflux 30 minutes on the water bath. Part of the amine is dissolved and traces of other ethyl compounds are decomposed; a

pale green color that may be formed with atmospheric oxygen also disappears. Immediately before use shake so as to hold the fine crystals in suspension.

PROCESS. To a portion of the flour or bread in a test tube, add *naphtha*, shake well or disintegrate with a rod, then add the *reagent* and shake again. If more than 1 : 10,000 parts of benzoyl peroxide is present, a green color forms immediately; if 1 : 20,000, it forms in a few minutes.

Simplified Modification. The test may also be performed as follows. Fold a lignin-free filter paper in half, wet with the *reagent*, and sprinkle the flour over half of the paper, then fold the other half over the flour and press with a glass plate. If benzoyl peroxide is present, blue-green dots (or a diffused coloration) appear on the paper.

NOTE. An anonymous author's procedure follows.[105] Spread out 5 to 6 g. of flour on a glass plate, smooth the surface, and add a few drops of *1% benzidine solution.* A green coloration at the edges indicates the residue derived from benzoyl peroxide bleaching.

Mohler-Illing Hydroxylamine Colorimetric Test. In the Illing modification [106] of the Mohler test for benzoic acid, the solution containing the benzoate is evaporated in a test tube in a boiling salt bath, then cooled and treated with 0.1 g. of *potassium nitrate* and 1 ml. of *sulfuric acid*, and finally heated in a boiling water bath for 20 minutes. After cooling, 2 ml. of water are added, then while the tube is held under the tap 10 ml. of *15% ammonia* and 2 ml. of *2% hydroxylamine hydrochloride* are added and the color is developed at 65° in 5 or 6 minutes. The color is matched against a solution of *ferric alum* treated with *potassium thiocyanate.*

Scott Modification.[107] As adapted, the steps of the method for the detection of benzoyl peroxide bleaching are as follows: (1) extraction of 500 g. of the flour with 800, 200, and 200 ml. of *ethanol*, (2) evaporation in the presence of *sodium hydroxide*, (3) distillation after acidifying with *sulfuric acid*, (4) decolorization of the distillate with *stannous chloride*, (5) distillation again in the presence of *acid*, repeating if necessary, and (6) application of the Mohler-Illing test.

Nicholls Ferric Chloride Colorimetric Method.[108] Nicholls (Government Laboratory, London) separates by distillation the benzoic acid formed from the benzoyl chloride during bleaching and oxidizes to salicylic acid, which is determined colorimetrically after ferric chloride solution is added.

REAGENTS. *Acid Ferric Chloride Solution.* Dilute 2.7 g. of $FeCl_3 \cdot 6H_2O$ and 13 ml. of 1.0 N H_2SO_4 to 100 ml.

Salicylic Acid, 0.01%. Standardize against known amounts of benzoic acid treated as in the actual determination.

PROCESS. *Distillation.* Distil with steam a mixture of 100 ml. of water, 50 g. of the flour, 40 g. of *calcium chloride*, and 10 ml. of *hydrochloric acid* and collect 300 ml. of distillate.

Ether Extraction. Remove the distillate to a separatory funnel, saturate with *sodium chloride*, and extract with two 50-ml. portions of *ether*. Evaporate the ether from the extract at room temperature in a rapid air current.

Oxidation. Add 5 ml. each of *acetone* and *2 N sodium hydroxide solution*, dilute with an equal volume of water, and evaporate the acetone by boiling. Cool the solution to 50 to 60°, add *potassium permanganate* in excess, then 10 ml. of *2 N sulfuric acid*, and reduce the excess of manganese oxide by *oxalic acid.*

Ether-Naphtha Extraction. Filter off any insoluble fatty acids, then extract with two 20-ml. portions of a mixture of equal parts of *ether* and *naphtha* and evaporate the extract to dryness at 30° in an air current.

Color Formation. Dissolve the residue in 15 ml. of warm water, cool, and add 1 ml. of *acid ferric chloride solution* and 1 ml. of *0.1% hydrogen peroxide solution.* Heat to 100°,

add 0.5 ml. of *1.0 N sodium hydroxide solution*, and filter while hot, washing with hot water to a volume of 50 ml.

Color Comparison. To the cold filtrate, add 1 drop of *acid ferric chloride solution* and match the color against 15-ml. portions of water, treated as in the actual analysis, to which *0.01% salicylic acid* has been added in serial amounts.

Munsey Hydroxylamine Colorimetric Method.[109] The benzoic acid is removed from the flour by steam distillation with *acid* and *salt* and the distillate is oxidized with *permanganate*, decolorized with *oxalic acid*, and extracted with *ether-naphtha mixture*, these steps being essentially the same as in the Nicholls method, except that calcium chloride is omitted. Then the extract, after removal of the solvent, is treated with *potassium nitrate* and *sulfuric acid*, neutralized, and the red color indicative of benzoic acid is developed by the addition of *hydroxylamine hydrochloride*.

Scott Modification.[110] In this modification, tentatively adopted by the A.O.A.C., calcium chloride is added to the distillation mixture as in the Nicholls method and the treatment with potassium permanganate and oxalic acid of the Munsey method is replaced by extraction with amyl alcohol and oxidation with hydrogen peroxide.

A. Qualitative Process. *Distillation.* To 250 ml. of water in a short-necked 800-ml. Kjeldahl flask, add 125 g. of the sample, 100 g. of *salt*, and 80 g. of *dried calcium chloride*. Shake and steam-distil 325 ml. as rapidly as possible after the initial foaming.

Ether Extraction. Saturate the distillate with 100 g. of *salt*, transfer to a 500-ml. separatory funnel, and extract twice each with 50 ml. of *ether*. Evaporate the combined ether extract at room temperature in a crystallizing dish with the aid of a fan, dissolve the residue in 5 ml. of *acetone*, add 7 ml. of *2 N sodium hydroxide solution*, and transfer to a 150-ml. beaker, rinsing with 35 to 40 ml. of water. Heat cautiously, then boil for 20 minutes to remove the acetone, adding water to keep a constant volume.

Amyl Alcohol Extraction. Transfer while hot to a separatory funnel, extract with 25 ml. of *amyl alcohol*, and draw off the lower layer into a separatory funnel. Extract again, using 20 ml. of *amyl alcohol*, and draw off the lower layer into a 250-ml. casserole. Combine the amyl alcohol solutions, add an equal volume of *naphtha*, extract 3 times with 5 ml. of water, and add to the combined aqueous extracts in the casserole. Reject the amyl alcohol-naphtha solution.

First Peroxide Treatment. Add to the aqueous solution 2 ml. of *30% hydrogen peroxide* (Superoxol), heat slowly to boiling, and boil until foaming ceases. Cool, acidify to *litmus* with *1 + 1 sulfuric acid*, and pour into a small separatory funnel.

Ether-Naphtha Extraction. After cooling, extract twice with 20 ml. of a mixture of equal parts of *ether* and *naphtha*, collect the extracts in a large test tube, add 2 ml. of *2 N sodium hydroxide solution*, stopper, shake, add a bit of thread to insure even boiling, and evaporate, at first slowly, over steam.

Second Peroxide Treatment. Heat in a vigorously boiling saturated salt solution, add a drop of *peroxide solution*, then when foaming ceases another drop, continuing the dropwise addition until the solution is almost colorless. If the evaporation is too rapid, add a drop or two of water. Finally evaporate to complete dryness and heat at 100° *in vacuo* for about 30 minutes.

Nitrate Treatment. After cooling, add 0.3 g. of *potassium nitrate* and 3 ml. of *sulfuric acid*, and heat in boiling water for 20 minutes, using a rod to incorporate all solid matter. Cool and add 6 ml. of water with stirring.

Color Formation. Add 15 ml. of *ammonium hydroxide* slowly with stirring, then 2 ml. of *6% hydroxylamine hydrochloride solution*, and heat in water at 65° for 5 to 6 minutes with occasional stirring. Cool, filter into

another tube, and observe the color, which, if red, indicates the presence of benzoic acid.

B. SEMI-QUANTITATIVE PROCESS. Prepare a series of Nessler tubes containing 0.2 to 1.5 mg. of *benzoic acid in ether solution;* 1 mg. = 1 ml. To each add 2 ml. of *2 N sodium hydroxide solution*, shake, and proceed as above, beginning with *second peroxide treatment*.

Color Comparison. Match the color of the unknown against one of the series of knowns.

CALCULATION. To obtain the approximate content of benzoic acid in gammas per gram, multiply the sample reading in milligrams by 32. This factor is based on 125 g. of sample and a minimum recovery of 25% of benzoic acid.

PERSULFATES

(Persulfate Bleaching)

Qualitative Test.[111] Spread 5 to 6 g. of the sample on a glass plate, smooth the surface, and add a few drops of *1% potassium iodide solution*. A blackish coloration indicates persulfates.

BROMINE

(Bromine Bleaching; Yeast Food)

No evidence that bromine has been used commercially for bleaching has come to the writers' attention. Any results of tests showing the presence of bromine in prepared flour or yeast may be explained as due to the addition of yeast food containing bromates or other bromine compounds.

Denigès and Chelle Fuchsin Test.[112] REAGENTS. *Fuchsin Reagent.* Add 10 ml. of 1 + 1000 fuchsin solution to 100 ml. of 1 + 20 H_2SO_4 and allow to stand 1 hour or until colorless.

Oxidizing Mixture: $H_2SO_4 + K_2Cr_2O_7$.

PROCESS. Treat the extract with *oxidizing mixture* and adsorb the liberated bromine in *sodium hydroxide solution*. Mix 25 ml. of *fuchsin reagent*, 25 ml. of *glacial acetic acid*, and 1 ml. of *sulfuric acid*. To 5 ml. of the mixture, add 1 or more drops of the solution of the unknown. If bromine is present, there is formed a red substance which may be dissolved in *chloroform* and compared with a *standard solution* obtained in like manner.

Bisulfite Test.[113] In the absence of persulfates, as shown by the method given below, add a drop of *0.25 N sulfuric acid* containing 4 drops of *sodium bisulfite solution* (36° Bé.) per 100 ml. A black spot coloration indicates bromates. In the presence of persulfates, add to a fresh portion *rosaniline bisulfite solution* (a 0.1% fuchsin solution, decolorized with a minimum amount of sodium bisulfite). A carmine red coloration indicates bromine.

Hahn Fluorescein Test.[114] REAGENTS. *Fluorescein Reagent.* Dissolve 0.1 g. of fluorescein in 5 ml. of 0.1 N NaOH solution and dilute to 1 liter.

Acetate Buffer Solution. Prepare approximately normal solutions of NaOH and acetic acid. Determine their relative value by titrating a small measured portion of the alkali solution with the acid, then add to the main portion of alkali solution an equivalent volume of the acid and enough more to obtain a pH of 5.5 to 5.6.

Chloramine Solution, 0.1 N. Dissolve 2.816 g. of the crystalline salt (chloramine T, sodium *p*-toluenesulfonchloramide) in 100 ml. of water.

Alkaline Thiosulfate Solution. Dissolve 1 g. of $Na_2S_2O_3 \cdot 5H_2O$ and 10 g. of NaOH in 200 ml. of water.

PROCESS. *Color Formation.* To 1 ml. of an extract of the flour, add 1 drop of the *fluorescein reagent*, then 3 drops of the *acetate buffer solution* and 1 drop of *0.1 N chloramine solution*. After 1 to 1.5 minutes, arrest the oxidation by adding 1 drop of *alkaline thiosulfate solution*.

Color Comparison. Match the color against

standard bromate solutions, containing 0.1 to 2.0 γ of bromine as bromate, treated in like manner.

Yates Chromic Acid-Iodide Volumetric Method.[115] The method, as originally developed for blood analysis at the Cardiff City Mental Hospital, is briefly as follows. Remove the protein by precipitation with *tungstic acid*, treat an aliquot of the filtrate with *potassium hydroxide*, evaporate to dryness, and heat at 500° for 20 minutes in a nickel crucible. Take up the residue, acidify, and oxidize the bromide to bromine by *chromic-sulfuric acid mixture*. In this concentration the chloride remains unoxidized, whereas the bromide is oxidized completely.[116] Remove the bromine by aeration and absorption in *starch-iodide solution*, then titrate the liberated iodine with *0.001 N sodium thiosulfate solution* delivered from a micro buret.[117]

Geddes and Lehberg Modification.[118] The Kulman flotation method and two colorimetric methods were found to be unreliable, but the modified Yates method gave recoveries of 93 to 97% from flours containing 0.001 to 0.005% (10 to 50 γ/g.) of potassium bromate. Direct leaching with water gave better results than leaching after ashing. Chlorates, iodates, and persulfates do not interfere.

PROCESS. *Incineration.* Leach 20 g. of the sample in water and incinerate with *potassium hydroxide*.

Oxidation and Titration. Take up the ash with water and oxidize with the *chromic-sulfuric acid mixture* in a special flask, then aerate into *starch-iodide solution* and titrate with *0.001 N thiosulfate solution*.

Ibanez Dichromate-Fuchsin Colorimetric Method.[119] APPARATUS. *Colorimeter.*

REAGENT. *Denigès-Chelle Reagent.* Mix 100 ml. of 5% by volume H_2SO_4 with 10 ml. of 0.1% fuchsin solution.

PROCESS. *Solution.* Weigh 10 g. of flour or finely ground bread into a 200-ml. Erlenmeyer flask, add exactly 50 ml. of *90 to 95% ethanol* and exactly 2 ml. of *10% sodium hydroxide solution*, then weigh the flask and contents. Heat to boiling over metal gauze or in a boiling water bath with shaking, allow to stand for a time, and boil again. Replace the ethanol lost by evaporation as determined by weighing, allow to settle for a moment, and decant the clear liquid.

Evaporation and Ignition. Remove exactly 26 ml. of the solution (= 5 g. of the sample, allowing for the expansion of the hot ethanol) to a 50- to 60-ml. porcelain crucible, evaporate, ignite at a dull red heat, cool, add 5 to 8 drops of *10% sodium hydroxide solution*, and again ignite to a nearly white ash.

Color Formation (Denigès-Chelle Reaction). After cooling, dissolve the ash in exactly 10 ml. of water, centrifuge to remove carbon, pipet 5 ml. into a test tube, and add 5 drops of *10% potassium dichromate solution* and 1 ml. of *sulfuric acid*. Cool for 15 minutes in water at 15 to 20°, then add 1 ml. of the *Denigès-Chelle reagent* and 1 ml. of *washed chloroform*. Close with a rubber stopper, mix, allow to stand with occasional shaking until the maximum color intensity of the chloroform solution has been reached.

Color Comparison. Compare with a *standard bromide solution* treated with *dichromate, sulfuric acid,* and *Denigès-Chelle reagent* exactly like the sample.

EXAMPLES. Wheat 1 to 7.5, wheat flour 3 to 4 (5.5 in one case) γ/g. of combined bromine; bread, amounts equivalent to the bromine added in the flour and salt. Data on products to which bromine has been added as a yeast food or bleaching agent are not available.

ODOR AND TASTE

Of primary importance to the expert in grading flour are the senses of odor and taste, which confirm or extend the conclusions reached by certain other simple physical tests.

The nostril is an especially delicate organ

in detecting certain forms of deterioration of flour, such as mustiness. Taste is a criterion in bread examination. The flour examiner, like the tea taster, speedily reaches a practical conclusion. Flavor due to crust formation or added ingredients, such as malt, should not be confused with natural flavors inherent in the grain, although the latter are too often overlooked in attempts to please the eye.

Flour experts lay stress on the granulation as determined by rubbing the flour between the thumb and fingers. Gritty flour feels coarse and harsh to the touch, owing to coarse particles; smooth flour feels soft because of fine grinding.

So far as the writers are aware, no instrument for testing the granulation is available, although such an instrument could be based on the degree of friction exerted by a weighed amount of flour between two disks.

Grittiness and smoothness are not due solely to degree of milling. Considering the structure of the kernel, the hard "horny" part of the endosperm becomes smooth only by fine grinding, whereas the floury part yields smooth flour with slight grinding. It is evident that the ratio of "horny" to floury endosperm must play an important part in the regulation of milling machinery and in determining the character of the finished product.

Dough

Dough Test. The determination of gluten by washing the dough is an extension of the dough test long practiced by miller, merchant, and baker who acquire no little skill in judging the suitability of the flour for different types of oven products. The flour seller "on 'change," in addition to displaying his samples, provides a pitcher of water for the use of his customer in making the dough test.

Absorption

Work up 30 g. of the sample with a spatula in a coffee-cup or bowl with 15 ml. of water so as to form a smooth ball of dough. Let stand 2 minutes and examine the character of the dough. If too dry, start with a new portion of flour, using 5 ml. more of water than before, and continue the trials until a dough of suitable texture and elasticity is obtained. Calculate the results to milliliters of water per kilo of flour.

Expansion

Rub in a mortar to a smooth paste 3.5 g. of *sugar*, 1.2 g. of *salt*, and 3 g. of *compressed yeast*, then mix well with 60 ml. of water at 35°. Add the mixture to 100 g. of the sample previously warmed to 35° in a pan, mix with a spatula, and knead to a smooth dough with the fingers. Transfer the mass to a graduated 500-ml. cylinder in such a manner as to form a compact mass free from air and with a flat surface. Note the volume, raise in a closet at 35°, and read the volume after 1 hour and again at half-hour intervals thereafter.

Apparent Viscosity

Bayfield-Harrel Viscometer Method.[120] Apparatus. *MacMichael Viscometer*, with 7-cm. bowl, 2.375 ± 0.01-in. (6.033 ± 0.025-cm.) disk plunger, 0.25 ± 0.005-in. (0.635 ± 0.013-cm.) clearance between bottom of disk and inner surface of bowl as measured by depth gage with 0.001-in. (0.003-cm.) divisions, and No. 30 viscometer wire. Keep the instrument level and the bob free from the sides; adjust dial to zero. Run at 12 r.p.m. as checked frequently by a stop-watch to guard against overspeed due to warming.

Reagent. *Lactic Acid.* Prepare from concentrated lactic acid a solution slightly stronger than normal, reflux for 3 hours, cool, and add water until normal or, as proposed by Reiman,[121] heat in an Erlenmeyer flask at

80° under an air condenser for 24 hours an approximately 0.85 N solution, as standardized with 0.01 N NaOH, and dilute until exactly normal.

PROCESS. *Preparation of Flour-Water Suspension.* Place in a clean, dry 500-ml. Erlenmeyer flask a quantity of flour equivalent to 20 g. on the 15% water basis, add 100 ml. of water at 30°, close with a rubber stopper, and shake vigorously 1 minute. Heat in a constant temperature cabinet or water bath at 30° for 1 hour, shaking about 10 times every 15 minutes. Remove from the heat, add 3 to 4 drops of *caprylic alcohol*, shake 10 times to remove foam, and pour into the viscometer bowl.

Direct Reading. Pour the suspension into the viscometer bowl, then make sure the bowl is flush on its supports. Start the machine and stir the solution with the bob 25 times, to insure a uniform suspension, before placing the bob or disk in position, then place the wire of the bob in its holder, damp swing of dial by placing a finger on the indicator pointer, then gradually touch the swinging dial and take a reading.

Reading After Acidulation. Add 1 ml. of *normal lactic acid solution* and take a second reading, then other readings after addition of 2-ml. increments of *normal lactic acid*, keeping the motor in operation between readings. After or during each addition of lactic acid, stir by moving the bob up and down 25 times, suspend the bob by wire, and read.

CALCULATION. Determine the maximum apparent viscosity of the acidulated flour-water suspension by plotting the apparent viscosity readings against the volume of acid, which is added in 2-ml. increments until no further increase is noted, the total being about 7 ml.

DIASTATIC ACTIVITY

Rumsey-Blish and Sandstedt Ferricyanide Volumetric Method.[122] REAGENTS. The reagents differ in minor details from those for the Sandstedt method for sugars. A revision whereby the same reagents can be used for both methods seems desirable.

Acid Buffer Solution, pH 4.6 to 4.8. Dilute 3 ml. of glacial acetic acid and 4.1 g. of anhydrous sodium acetate to 1 liter.

Standard Ferricyanide Solution, alkaline, 0.05 N. Dissolve 16.5 g. of $K_3Fe(CN)_6$ and 22 g. of anhydrous Na_2CO_3 in water and dilute to 1 liter. Keep in dark bottles in a dark place.

Standard Thiosulfate Solution, 0.05 N. Dissolve 12.41 g. of $Na_2S_2O_3 \cdot 5H_2O$ in twice-distilled CO_2-free water, the second distillation being made with the addition of a little alkaline $KMnO_4$. Thus prepared, it keeps well. A mixture of 10 ml. of 0.05 N $K_3Fe(CN)_6$, 25 ml. of acid-salt solution, 1 ml. of 50% KI solution, and 2 ml. of soluble starch solution should require 10 ml. of 0.05 N $Na_2S_2O_3 \cdot 5H_2O$ to discharge the blue color. Standardization as directed under Iodine Number (Part II, B2) is also desirable.

Acid-Salt Solution. Dilute a mixture of 200 ml. of glacial acetic acid, 70 g. of KCl, and 20 g. of $ZnSO_4 \cdot 7H_2O$ to 1 liter.

Potassium Iodide Solution. To a colorless 50% solution add 1 drop of 1 + 1 NaOH solution for each 100 ml. to prevent deterioration.

Soluble Starch Solution, 1% in 30% sodium chloride. Pour slowly a suspension of 1 g. of soluble starch in water into boiling water, then add 30 g. of NaCl and dilute to 100 ml. The solution should be colorless and transparent.

PROCESS. The following instructions apply to ordinary flour in which the maltose produced in 1 hour will seldom if ever exceed 350 mg. If the method is applied to material such as products from malted or sprouted cereals, use 1 to 3 ml. of the extract instead of 5 ml. and apply a corresponding calculation factor.

Digestion. Mix by rotating in a 100- or 125-ml. Erlenmeyer flask 5 g. of the flour with a teaspoonful of *quartz sand*, then with 46 ml. of *acid buffer solution*, each previously heated to 30°, until all the flour is in complete suspension. Digest 1 hour in a bath at 30°, rotating every 15 minutes.

Tungstate Precipitation. Remove from the bath, add 2 ml. each of *3.58 N sulfuric acid* and *12% sodium tungstate solution*, mixing thoroughly after each addition. Allow to stand 1 to 2 minutes and filter preferably through a No. 4 Whatman paper, discarding the first 8 to 10 drops.

Ferricyanide Treatment. Without delay pipet 5 ml. of the extract and 10 ml. of *standard 0.05 N ferricyanide solution* into a 50-ml. test tube (18 to 20 mm. diameter) and immerse in vigorously boiling water so that the containing liquid is 3 to 4 cm. below the surface of the water on the bath. After digesting exactly 20 minutes, cool the test tube under the tap and pour the contents into a 100- or 125-ml. Erlenmeyer flask, rinse with 25 ml. of *acid-salt solution*, and mix thoroughly.

Titration. Add 1 ml. of *50% potassium iodide solution* and 2 ml. of *starch solution*, mix thoroughly, and titrate with *0.05 N thiosulfate solution*, added from a 10-ml. micro

MALTOSE FROM FERRICYANIDE (BLISH AND SANDSTEDT)

0.05 *N* Ferricyanide	Maltose Equivalent	0.05 *N* Ferricyanide	Maltose Equivalent	0.05 *N* Ferricyanide	Maltose Equivalent	0.05 *N* Ferricyanide	Maltose Equivalent	0.05 *N* Ferricyanide	Maltose Equivalent
ml.	mg.	ml.	mg.	ml.	mg.	ml.	mg.	ml.	mg.
0.1	0.2	2.1	3.4	4.1	6.6	6.1	9.9	8.1	13.2
0.2	0.3	2.2	3.6	4.2	6.8	6.2	10.0	8.2	13.4
0.3	0.5	2.3	3.7	4.3	7.0	6.3	10.2	8.3	13.5
0.4	0.6	2.4	3.9	4.4	7.1	6.4	10.4	8.4	13.7
0.5	0.8	2.5	4.1	4.5	7.3	6.5	10.5	8.5	13.9
0.6	1.0	2.6	4.2	4.6	7.5	6.6	10.7	8.6	14.0
0.7	1.1	2.7	4.4	4.7	7.6	6.7	10.9	8.7	14.2
0.8	1.3	2.8	4.5	4.8	7.8	6.8	11.0	8.8	14.4
0.9	1.5	2.9	4.7	4.9	7.9	6.9	11.2	8.9	14.6
1.0	1.6	3.0	4.9	5.0	8.1	7.0	11.3	9.0	14.8
1.1	1.8	3.1	5.0	5.1	8.3	7.1	11.5	9.1	15.0
1.2	1.9	3.2	5.2	5.2	8.4	7.2	11.7	9.2	15.2
1.3	2.1	3.3	5.3	5.3	8.6	7.3	11.8	9.3	15.4
1.4	2.3	3.4	5.5	5.4	8.7	7.4	12.0	9.4	15.6
1.5	2.4	3.5	5.7	5.5	8.9	7.5	12.2	9.5	15.9
1.6	2.6	3.6	5.8	5.6	9.1	7.6	12.3	9.6	16.1
1.7	2.8	3.7	6.0	5.7	9.2	7.7	12.5	9.7	16.5
1.8	2.9	3.8	6.2	5.8	9.4	7.8	12.7	9.8	17.0
1.9	3.1	3.9	6.3	5.9	9.6	7.9	12.9	9.9	....
2.0	3.2	4.0	6.5	6.0	9.7	8.0	13.0	10.0	....

buret, to the complete disappearance of the blue color.

CALCULATION. Subtract the number of milliliters of thiosulfate solution used in the titration from 10, thus obtaining the number of milliliters of ferricyanide reduced to ferrocyanide by reducing sugars.

From the table above find the number of milligrams of maltose corresponding to the milliliters of ferricyanide. Multiply the milligrams of maltose by 20 to obtain the milligrams of maltose per 10 g. of flour found in the 1-hour digestion; this is the *diastatic value.*

Blank. No blank is ordinarily necessary if the flour was milled from sound wheat, since the content of reducing sugars originally present is so small and so constant that it may be disregarded in routine testing.

If, however, there is doubt as to the soundness of the wheat or there is evidence that it had been frosted, sprouted, heat-damaged, or otherwise damaged, make a blank determination as follows. Place 5 ml. of flour and a teaspoonful of *quartz sand* in a 100- to 125-ml. Erlenmeyer flask, then add 48 ml. of *0.4% (by volume) sulfuric acid* (preferably precooled in ice water), shake thoroughly, allow to stand 2 minutes, then filter, preferably through a No. 4 Whatman paper. Proceed with 5 ml. as in the actual analysis and make the necessary correction.

NOTE. Popov [123] employs the above method with unimportant modifications.

BAKING CHARACTERISTICS

The general purpose flour, like the general purpose cow, is a vanished delusion. Special flours are milled for special purposes to meet the special requirements of the different flour products, notably white bread, rye bread, cake, pie, biscuit, and griddle cakes. These flours are no longer tested by the miller's wife in her kitchen oven or by the miller's crude method of preparing the dough and determining in a rough way its physical characteristics. The laboratories of the flour mill and the modern bakery are equipped for making baking tests with an accuracy comparable with that of a chemical analysis; they also have special machines that determine and record certain physical characteristics—automatons as it were—that work with scientific precision, eliminating human error and the personal equation. The results of scientific tests are used by the baker not merely to secure the greatest "number of loaves per barrel of flour," but also as a guide for the manufacture of oven products that meet the demands of his customers for uniformity and excellence.

A.A.C.C. Bread-Baking Test for Wheat Flour.[124] The details which follow are essentially as adopted by the Association except that only the hand method of punching and molding are given. Baking methods for rye bread, cake, biscuit, pie, and crackers are also given in *Cereal Laboratory Methods.*

A. General Baking Characteristics. APPARATUS. *Conventional Experimental Dough Mixer*, preferably of the Swanson type, suitably calibrated.[125]

Thermometers. For dough, A.A.C.C. 15 to 40°; for oven 100 to 260°.

Fermentation Bowls. Aluminum or graniteware oatmeal bowls, top diameter 14.5 cm., bottom diameter 5 cm., depth 6.5 cm.

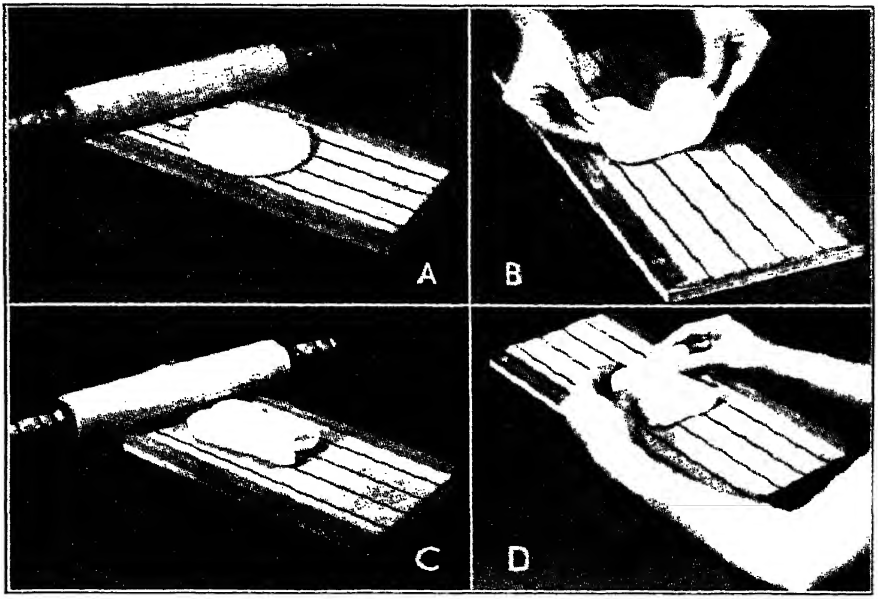
Fermentation Cabinet, capable of maintaining a temperature of 30 ± 0.5° and a relative humidity of at least 75%.[126]

Sheeter and Molder. In lieu of punching and molding machinery,[127] use hand punching technique (Fig. 98).

Baking Pans, 2xx tin. Low form, top 11.5 x 7.0, bottom 9.5 x 5.5, depth 5.0 cm.; tall form, top 10.5 x 6.0, bottom 9.3 x 5.3, depth at ends 6.8, at sides 8.5 cm.

Baking Oven, capable of maintaining a temperature of 230 ± 5°. A rotating plate or reel [128] is recommended.

Volume-Measuring Apparatus,[129] accurately calibrated by aluminum loaf models.



Courtesy of Cereal Laboratory Methods, p. 113

FIG. 98. Hand Punching and Molding Technique.

Miscellaneous Apparatus. Rough and fine balances, scoops, spatulas, and chemical apparatus.

REAGENTS. *Yeast Suspension.* Suspend for each series of tests 12 g. of fresh refrigerated compressed yeast in water and make up to 100 ml.

Salt-Sugar Solution. Dissolve 4 g. of NaCl and 20 g. of sucrose and make up to 100 ml.

PROCESS. *Mixing.* Place in the mixing bowl a quantity of flour equivalent to 100 g. on a 13.5 or 15.0% moisture basis (86.5 or 85 g. dry matter). Add 25 ml. of *yeast suspension*, 1 g. of *salt*, and an amount of *sugar* in suspension or solution estimated from the diastatic activity or gassing power as determined by special methods, or if preferred use 25 ml. of the *salt-sugar solution*. Finally add sufficient additional water to obtain the desired standard dough consistency, keeping all materials and apparatus at 30°. Multiples of the charge may be used.

Fermentation. Remove the dough from the mixing bowl and, if a multiple charge was used, scale to an appropriate weight either of dough (150 to 175 g.) or flour (100 g. on either 13.5 or 15.0% moisture basis). Round up by folding 20 times in the hand, place in the fermentation bowl, and ferment in the cabinet a total of 180 minutes. Punch after 105 and again after 50 minutes and mold after an additional 25 minutes.

Punching. If a punching machine is not available, mold by hand as follows. Place the dough, wet side down, on a piece of canvas belting, provided with a wooden track giving 5/16 in. (8 mm.) clearance (Fig. 98, *A*). With a rolling pin, roll once each way from the center of the dough. Invert the dough, overlap the opposite ends (Fig. 98,

B), replace, seam down, in the fermentation bowl, and return to the cabinet.

Molding. Sheet the dough as directed for punching above. Overlap the opposite ends of the dough sheet (Fig. 98, *B*), invert, and turn parallel with the longer axis of the wooden track. Roll once each way from the center (*C*), turn the dough over, and, starting at the more remote end, roll it up by hand toward the operator (*D*). Seal the seam, roll the dough lightly under the palm of the hand, and place, seam down, in the baking pans, limiting the length of dough to that of the pan prior to the final light rolling. Grease the pans *only* when absolutely necessary to prevent sticking.

Proofing. Proof the dough at 30° and at least 75% relative humidity, either to constant time (usually 55 minutes) or height (such as 9.5 cm.).

Baking. Place an open pan of water in the oven. Bake for 25 minutes at 230° (± 5°) with the oven thermometer at the level of the top of the baking pan, 5 cm. therefrom on the side next to the axis of rotation of the shelf. For better uniformity, bake dummy loaves before and after the actual tests.

Measurement and Scoring. Calculate the absorption (*A*) on a 13.5 or 15% moisture basis by the formula

$$A = W - (100 - F)$$

in which *W* is the total milliliters of water added and *F* is the weight of flour taken. In calculating the total water added, the 25 ml. of yeast suspension is equivalent to 22.5 ml. of water and 25 ml. of salt-sugar solution is equivalent to 21.5 ml. of water.

Weigh the loaf and measure its volume 1 hour after removal from the oven. Store in a tight cabinet until scored for crust color, symmetry, grain, and texture.[130]

Report method of proofing and molding, size of pan, diastatic compensation, etc.

B. Responses. (1) *Mixing Response.* Mix the dough for various lengths of time at 30° and note the changing characteristics of the loaves. Report the optimum mixing time and tolerance under specified conditions.

(2) *Fermentation Response.* Vary the fermentation time by increments above and below that recommended and note the character of the loaves. Report optimum fermentation time and tolerance under the specified conditions.

(3) *Bromate or Oxidation Response.* Add increments of potassium bromate in a series of tests and note the character of the resulting loaves. Report optimum amount of bromate and range of bromate tolerance in per cent, based on flour, under the conditions specified. The optimum bromate level for average flour seldom exceeds 0.005%.

A.A.C.C. Baking Tests for Rye Flour, Cake Flour, Self-Rising Flour, Pie Flour, Biscuit Flour, and Cracker Flour. See the last edition of the A.A.C.C. *Cereal Laboratory Methods.*

Koelner Straight Dough Bread-Baking Test for Wheat Flour. Although now chiefly of historic interest, the method when introduced marked an important step in the development of bread flour testing.

Apparatus. The *kneader* is really a combination of a *mixer and kneader* and is so used in the baking test. It consists of a metal container, set in an inclined position, in which revolve metal blades which may be adjusted for mixing or kneading. A special compartment for hot water holds the temperature at about 35°.

Fermentation (Rising or Proofing) Closet. A wall or desk cabinet with glazed doors.

Oven. Any type provided with pyrometer or thermometer for heats at 200 to 205°.

Baking Tins, 27 x 6.3 cm. at the top, 25.4 x 5 cm. at the bottom, and 8.8 cm. deep, all inside measurements.

Volume Measure. A box in which the largest loaf will fit without protruding. Calibrate as follows: Fill with flaxseed and carefully strike off the top with a straight-edge.

Weigh the flaxseed and divide the capacity of the box in milliliters by the grams of seed, thus establishing the equivalent of 1 g. of seed in milliliters of volume. In making the volume test, place the loaf in the box, fill with seed, and strike off the surface. Weigh the seed remaining in the box and multiply the weight by the equivalent of 1 g. in milliliters, then subtract that volume from the capacity of the box in milliliters, thus obtaining the loaf volume in milliliters.

This simple apparatus suffices for many purposes, but is less accurate and convenient than the National Manufacturing Company's volume tester.

Reagents. *Sugar, Salt,* and *Compressed Yeast.*

Process. *Tempering.* Warm at least 340 g. of the flour in a shallow pan in the fermentation closet kept at 35°. Transfer 220 g. of the flour to the Koelner kneader, which also has been warmed to about 35° by hot water in the special water compartment.

Mixing. Rub up to a smooth paste in a cup 12 g. of *sugar,* 5 g. of *sodium chloride,* 10 g. of *compressed yeast,* and 100 ml. of water at 35°. Add the paste to the flour in the kneader. Use for rinsing the cup sufficient water (usually about 87 ml.) to make the total volume required as calculated from the results of an absorption test. With the kneader blades adjusted for mixing, turn the crank of the kneader at 90 r.p.m. for 10 minutes.

Kneading. Add 120 g. of the flour at 35° and turn the crank at 60 r.p.m. for 10 minutes with the blades adjusted for kneading.

Fermentation. Cut the dough in half on a warmed plate, mold each half separately, place end to end in the baking pan, previously warmed, greased, and tared, place a tin gauge over the top, and allow to rise in the fermentation closet until the dough touches the gauge.

Baking. Place in the oven at 200 to 205° and bake for 30 to 35 minutes, until 30 g. have evaporated as determined by weighing.

Volume Test. Determine the volume in the volume measure.

Scoring. Note the odor while hot and after cooling; note the color and flavor when cold.

Long Fermentation Baking Test for Wheat Flour. The method embodies the features that bring out the special merits of bread flour of different characteristics, but is still not of universal application.

As Harry Snyder stated in a letter to the writers, "Each flour tested is entitled to the quantity and kinds of ingredients and method of manipulation as may be necessary to produce the best loaf of bread from a physical point of view, that the flour is capable of making."

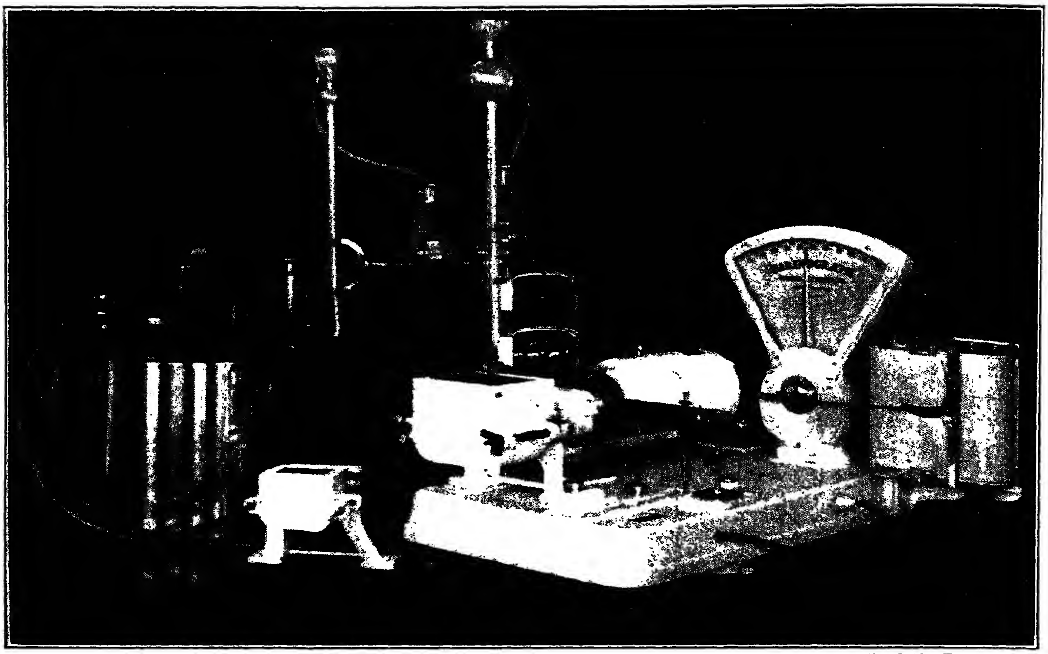
Apparatus. Same as for the foregoing method, except that the kneading may be done by hand.

Reagent. *Yeast Mixture.* Although the proportion of the ingredients must be varied to meet the conditions, the following is satisfactory for many purposes. Mix 9 g. of fresh compressed yeast, 3.5 g. of salt, and 12 g. of sugar with a suitable amount of water as calculated from the absorption test.

Process. *Tempering.* Place in a bowl 340 g. of the flour and 6 g. of *shortening,* then allow to heat at 32° in the fermentation closet together with the *yeast mixture* and water sufficient to make a stiff dough, the amount of water being regulated by the amount used in performing the absorption test.

Mixing. Make the flour, shortening, and yeast mixture into a dough.

Fermentation. Place in the fermentation closet where the temperature should be kept at about 32°. After 50 minutes, pull, knead, and return to the closet for 40 additional minutes, then pull, knead a second time, place in tins, and allow to rise for 30 to 50 minutes.



Courtesy of Brabender Corporation, Rochelle Park, N. J.

FIG. 99. Brabender Farinograph.

Baking. Heat the oven to 200 to 205° and bake the loaf.

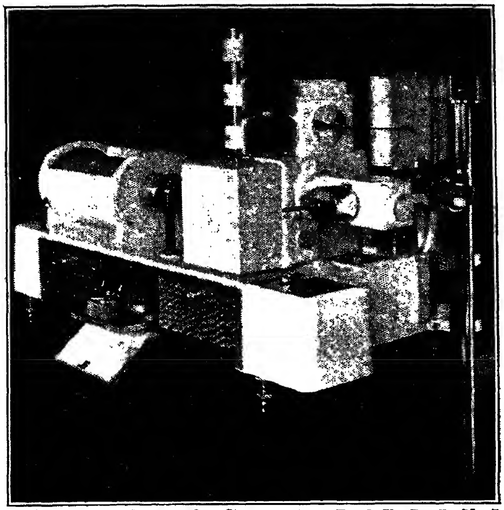
Scoring. As in the foregoing methods.

Machine Tests. As in the determination of moisture and gluten, accurate laboratory methods have replaced rough-and-ready mill or flour exchange tests, so also have ingenious precision machines been devised for furnishing more reliable information than by crude testing. Of the various testing machines which have been invented in both Europe and America, only the *farinograph*, which is given a place in the *A.A.C.C. Methods*, and its sister machine the *extensograph*, both supplied by the Brabender Corporation, Rochelle Park, N. J., are here illustrated and briefly described.

Farinograph.[131] The instrument (Fig. 99) simulates dough-mixing conditions and determines (1) absorption, (2) dough development or mixing time, (3) dough stability or mixing tolerance, and (4) after some practical experience in reading these curves, the type of wheat from which the flour was made. The conclusions drawn from the foregoing data for application in the bakery are: (1) strength and consequently approximate value of the flour in comparison with other flours, (2) mixing tolerance and handling properties, (3) actual mixing time, and (4) absorption. The farinograph tests the dough dynamically with the gluten strands in an excited state and tense condition.

Extensograph.[131] In contradistinction to the farinograph, the extensograph (Fig. 100) tests the dough while it is at rest and the gluten strands are relaxed, that is, the physical condition of the dough during fer-

mentation. It measures the extensibility of the dough and its resistance to extension, which are the most important factors responsible for (1) the loaf volume potentialities and (2) the maturing requirements of a flour. Furthermore, it gives information about such fermentation characteristics as (1) the comparative speed of fermentation in different



Courtesy of Brabender Corporation, Rochelle Park, N. J.

FIG. 100. Brabender Extensograph.

doughs, (2) the effect of different ingredients (e.g., different types of dried skim milk, or of malt, etc.) on fermentation characteristics, and (3) the fermentation tolerance of different flours, in addition to the loaf volume potentialities and maturing requirements mentioned above.

Bran

The difference in the percentage of bran in whole wheat flour and the grades of white flour collectively is brought out by the content of crude fiber, but the distinction of the different grades of white flour requires a more accurate method for which search has long been made.

Nottin and Lemoigne Pyrocatechol Test.[132] PROCESS. To a cup of flour, add a *solution of pyrocatechol* and mix to a homogeneous paste. The proportion of flour to reagent may vary widely without vitiating the results. During 30 minutes of standing, the flour takes on a more or less decided rose tint, depending on the bran content. Pyrocatechol gives more satisfactory differentiation than phenol, guaiacol, hydroquinone, pyrogallol, gallic acid, diaminophenol, and tyrosine. No great accuracy is claimed for the test.

Corn Flour

In Italy the addition of 10% of corn (maize) flour to wheat flour is required by law. Its presence and approximate determination are readily accomplished by the microscopic examination involving the form and relative number of the starch grains. The following chemical method is stated to give results within 0.25% of the truth.

Giuliani and Riparbelli Alkali Colorimetric Method.[133] PROCESS. Treat 2 g. of the sample with 25 ml. of *0.5% sodium hydroxide solution* for 12 hours with occasional shaking. Filter, add 2 ml. of *acetic acid*, evaporate to 2 to 4 ml., and compare the color with that of *standard solutions* prepared with known percentages of corn flour.

Soy Bean Flour

LaWall and Harrisson Tests.[134] **A. Qualitative Urease Test.** In a small test tube, mix 0.5 g. of the sample with 5 ml. of 2% *urea solution* and partly immerse a piece of *litmus* or *bromothymol paper*. Stopper and heat at 40° for 3 hours. An appreciable amount of soy bean (or jack bean) flour produces a blue coloration which is due to the liberation of ammonia from the urea by the urease. The test is not applicable to products containing leavening material.

B. Semi-Quantitative Urease Test. Mix in a test tube 150 mg. of the sample with 10

ml. of *1% urea solution*, stopper, keep at 25° for 30 minutes, then titrate directly with *0.1 N sulfuric acid*, using *methyl orange indicator*. From the following data, some idea may be formed as to the content of urease in the various products: cereals 0.1 to 0.2, soup beans 0.6, Lima beans 0.75, French beans 0.55, Greek beans 0.65, jack beans 33.13, soy beans 17.90 to 33.10, and soy flour 3.10 to 10.40 ml. of 0.1 N acid.

C. Hanging-Drop Tests (for **Peanut Butter**). Moisten 2 g. of the defatted sample in a porcelain capsule with *2% urea solution*. Cover with a microscopic slide with a hanging drop of *10% hydrochloric acid* and let stand in a warm place overnight. The presence of soy bean is indicated by a marked crystalline residue of ammonium chloride.

D. Potassium Hydroxide Test. Make a stiff paste with the flour and *5% potassium hydroxide solution*. A yellow color appears immediately if soy bean flour is present.

E. Naphtha Test. Shake 1 g. of the sample with 10 ml. of *naphtha*. A yellow supernatant liquid and a yellow sediment appear with soy flour. Wheat flour, containing 5% of soy flour, gives a spotted sediment.

F. Hydrochloric Acid Test. Make a paste with the flour and *hydrochloric acid*. Soy flour gives a lavender color, as does also wheat flour, but more slowly.

G. Microscopic Examination of a mixture of wheat flour and soy flour, after treatment with *iodine solution*, distinguishes the wheat particles (blue) from the soy flour (yellow). Further microscopic examination shows the characteristic hour-glass cells.

3. BREAD

Cellulose

Kürschner and Hanak Acetic-Nitric Acid Gravimetric Method.[135] Originally designed for cocoa products, the method, slightly modified, has been shown by Ladd [136] to be well suited for estimating the content of whole wheat flour in oven products.

Apparatus. *Digestion Flask.* A 30- to 35-ml. Jena flask with a ground-in condenser tube.

Process. *Digestion.* Weigh 0.3 g. of the (not defatted) sample into the digestion flask and add 15 ml. of *80% commercial acetic acid* and 1.5 ml. of *nitric acid*. Mix well with a rotatory motion and boil for 20 to 25 minutes over a small flame.

Filtration. While still hot, filter on a Gooch crucible previously moistened with *acetic acid*. Wash with stirring in the ordinary manner with the following liquids added first to the flask to aid in removal of the fiber: (1) 7 to 10 ml. of the hot *digestion mixture*, (2) hot water, (3) a few drops of *ethanol* to moisten the asbestos, (4) 5 to 10 ml. of *ether*, (5) 1 to 2 ml. of hot *digestion mixture*, and (6) hot water to the complete removal of the acid odor. In adding the ether, take care to rinse the sides of the crucible.

Drying. Dry gradually and finally heat at 105 to 108°, cool, and weigh. The drying may be hastened by a final washing with *ethanol*, followed by *ether*.

Ladd Modification.[137] A larger quantity of material and a larger flask than in the original method are employed. Three digestion reagents were given trials as follows: (1) 60 ml. of *acetic acid* plus 1.5 ml. of *nitric acid* (sp.gr. 1.4) (the regular Kürschner and Hanak reagents), (2) the same as (1) but with the addition of 2 g. of *trichloroacetic acid*, and (3) the same as (1) with the addition of 1.5 ml. of *hydrochloric acid*. Although all gave satisfactory results, reagent (2) was preferred. Digestion for 30 minutes is recommended.

Nicotinic Acid

Swaminathan Aniline-Cyanogen Bromide Colorimetric Method. See Part I, C10, and Part II, A2.

Bina, Thomas, and Brown Modification.[138] PROCESS. As applied to bread proceed as follows.

Solution. Dry the sample at a low temperature, grind, suspend in water, and autoclave, then cool, hydrolyze by digestion with *taka-diastase,* followed after centrifuging by heating on a boiling water bath with *hydrochloric acid.* Cool and adjust the pH, then remove an aliquot.

Color Formation. Proceed as in the original Swaminathan method or one of its modifications.

IRON

See also A2 above.

Herapath-Winter Thiocyanate Colorimetric Method. See Part I, C8*b*.

Hoffman, Schweitzer, and Dalby[139] prepare the ash and solution as follows. Place 1 g. or more of the sample in a high quality silica ashing dish, add 5 ml. of *1.0 N sodium hydroxide solution* per gram of sample, mix well, dry at 100°, and ash at low redness.

Use for the preparation of the standard a *solution of iron wire* of highest purity.

MILK SOLIDS

Hoffman, Schweitzer, and Dalby Method.[140] These authors, working at the Research Laboratory of the Ward Baking Company, New York, found that the amount of lactose attacked by yeast is negligible, but that other reducing sugars are completely removed during fermentation.

A. Lactose. PROCESS. *Solution.* After removing the crust, air-dry the crumb and grind to pass a 20-mesh sieve. Digest 50 g. of the ground material in 400 ml. of water at 40° for 3 hours and centrifuge. Decant the supernatant liquid into a 1-liter volumetric flask and wash with 4 portions of 75 ml. each of water. Add to the solution 35 g. of *baker's yeast* suspended in water, 0.5 g. of *ammonium sulfate* (yeast stimulant), and 0.2 g. of *sodium bisulfite* (bacteria retardant). Allow to stand overnight, leaving a vent for the escape of gas.

Clarification. Add to the solution 20 ml. of *copper sulfate solution (Fehling-Soxhlet 1)* and sufficient *sodium hydroxide solution* to give a definite blue color and clarify the solution. Make up to volume, shake, and filter through a dry paper.

Copper Reduction. Determine lactose by the Munson and Walker gravimetric method in 50 ml. of the filtrate.

CALCULATION. To obtain the content of fat-free milk solids (dry basis), multiply the content of lactose (dry basis) by 2.

B. Whole Milk Solids. PROCESS. *Solution.* Place 200 to 300 g. of the finely ground air-dry sample in a 2-liter flask containing 1 liter of water and 30 ml. of *hydrochloric acid.* Boil for 1 hour or until the solution is well digested, then add 10 g. of *Filter-Cel.* Filter through a Büchner funnel fitted with a circle of filter paper over which is a pad of Filter-Cel, continuing the suction until the mass is fairly dry.

Fat Extraction. Transfer the residue to a beaker, stir with *ether*, and again filter through *Filter-Cel* into a dry flask. Evaporate the ether solution, dry the fat, and calculate the percentage.

Determination of Reichert-Meissl Number. Proceed as directed in Part II, B2.

CALCULATION. Multiply the content of fat-free milk solids by 0.4115, thus obtaining the content of fat necessary to balance the skim milk solids. Calculate also the butter fat content, assuming a Reichert-Meissl number of 28 and correcting the percentage of fat for 0.7 which is the average in flour, dry basis. Add the calculated per cent of fat-free milk solids to that of the butter fat to obtain the percentage of whole milk solids. If the Reichert-Meissl number indicates only part of the butter fat necessary to balance the skim milk solids, it may be concluded that partially skimmed milk was used.

Butter Fat and Fat Number

Munsey Fat Number Method.[141] The fat number is a value similar to the Reichert-Meissl number, but is made on one-fifth of the amount of fat and titrated with standard alkali of one-fifth of the strength.

Process. *Acid Treatment.* Place 50 g. of the dried, ground, and sifted (20-mesh) sample in a 600-ml. beaker. Add 100 ml. of water, mix, add 100 ml. of *hydrochloric acid*, mix, cover, and heat on the steam bath for 1 hour, stirring thoroughly 6 or 7 times. Cool in water at 15° or below, add 50 ml. of ice-cold water, stir, add 10 g. of *Filter-Cel*, and stir well. Collect the insoluble matter and Filter-Cel on a 90-mm. Büchner funnel fitted with two 9-cm. papers and a layer of 10 g. of *Filter-Cel*. Wash down the sides of the Büchner funnel with ice-cold water, apply suction and transfer the mass to the original beaker. Rinse off filter and funnel with *naphtha* and add the rinsings to the beaker. Break up the mass with a rod, heat on the steam bath to remove most of the water, then heat in an oven at 100° for 1 hour or until dry. Add 25 g. of *anhydrous sodium sulfate*, breaking up any lumps.

Ether-Naphtha Extraction. Macerate the mass in the beaker with 150 ml. of *1 + 1 ether-naphtha mixture* with a spatula for 3 or 4 minutes. Decant onto a filtering tube with an asbestos mat about 1 cm. thick, the stem of which passes through the stopper of a filtering bell glass into a 500-ml. Erlenmeyer flask, using suction. Repeat the maceration with 80 ml. of the *solvent mixture* and decantation, then transfer the mass to the tube, using 100 ml. of the *solvent mixture* for rinsing and washing. Repeat twice, add a piece of thread extending to the bottom of the flask, and concentrate the extract on the steam bath. Transfer to a tared 150-ml. flask, using *naphtha* for rinsing, dry at 100° for 30 minutes in an oven, cool, weigh, and calculate the percentage of fat, dry basis.

Determination of Volatile Fatty Acids. Weigh into a 300-ml. flask 1 g. of the fat and proceed as in the determination of the Reichert-Meissl number, using, however, *0.02 N sodium hydroxide solution* for the titration, and calculate the number of milliliters required for 1 g. of fat. Multiply by 1.1 and divide by the weight of fat taken to obtain the fat number.

Egg Yolk

Grossfeld Dual Determination Gravimetric Method.[142] See Alimentary Pastes below.

The usual formula involving lecithin phosphoric acid is regarded as the best index of egg content, but this may be supplemented by the formulas based on the unsaponifiable matter, if the fat contains a large proportion of egg fat.

In the latter case, allowance must be made for the baking fats, the process being as follows.

Process. *Extraction.* Treat 100 g. of the air-dry and ground sample for 6 hours with a *1 + 1 mixture of ethanol and benzene*. Evaporate the solution, add 400 ml. of water and a little *acetic acid*, then precipitate with 2 ml. of *15% potassium ferrocyanide solution* and 2 ml. of *30% zinc acetate solution*. Separate the precipitate, dry, extract the fat with *ether*, evaporate, dry again, and weigh. Use a portion of the fat to determine the kind of shortening.

Calculation. Obtain the per cent of egg fat (E) in the total fat by the formula

$$E = \frac{100(B - b)}{52 - b}$$

in which B is the weight in milligrams of the unsaponifiable matter in the unknown obtained by procedure B and b is the corresponding value for the baking fat.

The assumed value of b for butter fat is 6, for oleomargarine 8, for lard 2, and for egg yolk fat 52 mg.

The per cent of egg yolk in the sample (Y) is calculated by the formula

$$Y = 3.2E$$

NOTE. Owing to war conditions, the original paper is not available and the above details are based on the necessarily limited data given in *Chemical Abstracts*.

SODIUM CHLORIDE

Caldwell-Irvin Modification of the Volhard Volumetric Method.[143] Irvin, of the Red Star Yeast & Products Co., Milwaukee, confirms the findings of others that incineration without a fixative such as sodium carbonate or hydroxide, as directed in the A.O.A.C. and A.A.C.C. methods, results in escape of chloride, but this is averted by the use of sodium carbonate, as specified in the former for ashing plants.

PROCESS. *Incineration.* Mix well 3 g. of the air-dried crumb with 10 ml. of *5% sodium carbonate solution* in a platinum or porcelain dish, dry at 120 to 130° for 1 hour, and char at a dull red heat. Cool, cover with water, let stand a few minutes in a warm place, treat with *1 + 4 nitric acid,* and filter. Return the paper and charred carbon to the dish for reignition and acid treatment.

Titration. Combine filtrates and washings (total 125 ml.), add 25 ml. of *standard 0.05 N silver nitrate solution,* 3 ml. of *nitrobenzene,* and 1 ml. of *ferric indicator* (saturated ferric ammonium alum solution), shake for 30 seconds or more, and titrate the excess of silver nitrate with *standard 0.05 N potassium thiocyanate solution* to a faint reddish brown tint that persists for 3 to 4 minutes.

CALCULATION. Obtain the per cent of sodium chloride (S) by the formula

$$S = \frac{(A - K) \times 0.002923 \times 100}{W}$$

in which A is milliliters of silver nitrate solution, K is the milliliters of thiocyanate solution required, and W is the weight of the charge.

4. ALIMENTARY PASTES

LECITHIN-PHOSPHORIC ACID

Lecithin is the constituent of eggs that is of chief diagnostic value in the estimation of the proportion of eggs to flour in noodles and similar pastes. The determination of lecithin itself involves certain difficulties, but that of phosphoric acid in the form of lecithin, as obtained in an alcoholic extract, is readily carried out by the following method.

Juckenack Ethanol-Soluble Phosphoric Acid Method.[144] PROCESS. Extract 30 g. of the finely ground sample for 10 hours with *absolute ethanol* in a Soxhlet extractor covered with asbestos paper so that the inside temperature is between 55 and 60°. A few bits of *pumice stone* in the flask will prevent bumping. Transfer the ethanolic extract to a platinum or porcelain dish, add 5 ml. of *4% ethanolic potassium hydroxide solution,* evaporate to dryness on a water bath, and heat over asbestos until the residue is charred. Take up the residue with *dilute nitric acid,* heat, filter, and wash with hot water. Return the paper to the dish, burn to a white ash, treat as before with *dilute nitric acid,* filter, wash, and unite the two solutions.

Determine phosphoric acid by the molybdic method. See Part I, C8*a*.

EXAMPLES. Juckenack and Pasternack[145] found in noodles percentages of lecithin phosphoric acid as follows. No egg 0.0225, 1 egg 0.0513, 2 eggs 0.0786, and 3 eggs 0.1044%. The corresponding amounts of ash were 0.46, 0.57, 0.66, and 0.76%; of total phosphoric acid 0.23, 0.27, 0.31 and 0.35%; of ether extract 0.66, 1.56, 2.42, and 3.24%; and of protein 12.00, 12.99, 13.92, and 14.81%. All the results are on the dry basis.

Lipoids

See A2 above.

Unsaponifiable Matter

Direct Saponification Gravimetric Method.[146] Process. *Extraction.* To 10 g. of the material ground to pass a 20-mesh sieve, in a 500-ml. Erlenmeyer flask, add with shaking 30 ml. of *1 + 1 hydrochloric acid* and heat on the steam bath for 30 minutes with occasional shaking.

Saponification. While cooling the inclined flask under the tap, add carefully with shaking 30 g. of *potassium hydroxide pellets* at a rate that permits boiling but not spurting with loss of material. While hot, place on the steam bath, cover with a small watch glass, and heat for 3 hours with occasional swirling to wash down the sides. Cool until just warm, add 30 ml. of *ethanol* and 50 ml. of water, and mix well.

Ether Extraction. Add 100 ml. of *ether*, swirl vigorously for 1 minute, and transfer to a 500-ml. separatory funnel with 50 and 25 ml. of *ether*, rinsing the flask with 50 ml. of *1% potassium hydroxide solution*, with swirling. Continue the swirling 10 to 15 seconds, then proceed as with Eggs, Part II, H7, Direct Saponification Gravimetric Method, beginning with *Removal of Soap Solution.*

Cholesterol

Determine the sterol as directed for cholesterol by the Haenni ether-bromine volumetric method (Part II, H7). If the unsaponifiable matter from egg-free products or from any product is less than 0.23% (dry basis), add 10 mg. of pure *cholesterol* before applying the cholesterol method and correct accordingly.

Eggs

Grossfeld Dual Determination Gravimetric Method.[147] See Part II, B2, under Sterols, Hydrocarbons, and Total Unsaponifiable Matter.

In applying the method to pastes, Grossfeld employs procedures A and B based on the content of unsaponifiable matter and a third procedure (here for convenience designated procedure C), based on the content of lecithin phosphoric acid. The grams of naphtha-soluble matter in 100 g. of the sample by the first two procedures appears in the following formulas with the same designations as the procedure, except that the letters are in italics (A and B); also Y representing the egg yolk content in the product in terms of grams (nearest 10 g.) per kilo is introduced. On the basis of procedure A, the formula for calculating the content of egg yolk is

$$Y = 99A - 61 \tag{1}$$

and the second formula, based on procedure B, is

$$Y = 4.7B - 61 \tag{2}$$

When the calculation is based on the content of lecithin phosphoric acid (L) the formula is

$$3500L - 70 \tag{3}$$

Usually formulas (2) and (3) are used and the presence of paraffin added as an adulterant is confirmed by formula (1).

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B. FATTY FOODS

1. OIL SEEDS; OIL FRUITS; OIL NUTS; AND OIL CAKES

Edible vegetable oils and fats are obtained largely from seeds (including nuts) of widely differing species; olive oil, however, which is the salad oil par excellence, is made from the flesh of a fruit. The oil from the kernel of the pit is of inferior value. The avocado is one of the few other edible fruits containing oil in the succulent fruit tissues.

The cakes remaining after expressing the oil are valuable concentrated cattle foods which are subject to official inspection and analysis.

In the cottonseed and members of the rape (mustard) group, the edible portion is largely cotyledons, in the cocoanut it is largely endosperm, and in the linseed it is divided between the cotyledon and the endosperm. The hulls of linseed, rapes, and mustards have little food value, but their presence is an indication of the occurrence of ground seed or cake.

Microscopic Structure (Figs. 101 to 106). Only one common oil seed (peanut) contains starch. The soy bean, the other common oily legume, contains no starch, although its relatives in field and garden are rich in starch. Cottonseed, linseed, and sesame seeds, three of the world's leading oil seeds, belong to different families and have practically nothing in common in their varied and beautiful structures.

Members of the mustard family are distinguished by the structure of the seed coat (spermoderm) as shown in the illustrations. Charlock is distinguished from the true mustards by the carmine red color produced with acid chloral hydrate solution, as noted subsequently.

The cocoanut, also the oil palm fruit and nut (all starch-free) are remarkable for their fat crystals and aleurone grains. The Brazil nut contains perhaps the finest example of aleurone grains. The chestnut is a starch seed.

The *oil globules*, which are conspicuous in microscopic mounts of sections or powders of all oil seeds, must be removed by extraction with a suitable oil solvent before the cell forms and cell contents can be clearly seen. The *starch* naturally present in the peanut and cacao (these, although treated elsewhere, are oil seeds) and any starch that may be present in mixtures of ground oil cakes, such as mustard flour, with starchy material is clearly brought out in the extracted fat-free material. In addition, cautious clearing with 5% potassium hydroxide or chloral hydrate solution is often desirable if only the cellular structure is studied.

Aleurone grains are best studied after mounting in oil which, however, interferes with the application of special reagents, such as sulfuric acid for gossypol or chloral hydrate for the coloring matter of the cells of charlock.

LEGENDS OF ILLUSTRATIONS ON THE FACING PAGE

The illustrations show the microscopic structure of five common oily fruits and seeds and charlock, an oily weed-seed produced in enormous quantities in the grain fields of the United States. The magnification is ×160.

FIG. 101. Cottonseed. Elements in surface view. Seed coat (hulls): *ep* outer epiderm with stoma, hair, and hair scar, *br* brown subepiderm, *w* white cells, *pal* palisade cells at two foci and on side, and *a* and *c* inner brown layers. *N* perisperm. *E* endosperm. Cotyledon: *aep* outer epiderm with multicellular hair and immature stomata, and *mes* mesophyl with crystal, aleurone grains, and resin cavity lined with thin-walled cells.

Cottonseed hulls, with their highly characteristic hairs and palisade cells, have no marked food value. The striking elements of cottonseed meal are the dark resin cavities, the contents of which turn brilliant red on the addition of sulfuric acid.

FIG. 102. Linseed. Below, fruit-coat tissues (hulls) in surface view: *epi* epicarp, *cr* crystal cells, and *end* endocarp. Above, left, seed in cross section: *S* seed coat, *E* endosperm, and *C* cotyledon; right, seed elements in surface view: *ep* mucilaginous outer epiderm, *r* round cells, *f* longitudinal fibers, *tr* transverse fibers, and *pig* pigment cells. *E* endosperm with aleurone grains. *C* outer epiderm of cotyledon with immature stomata.

The worthless hulls occur in linseed meal or cake. The meal is characterized by the mucilaginous epidermal cells, the crossing thick- and thin-walled fibers, and the more or less quadrilateral pigment cells with dark contents which often escape as solid angular masses.

FIG. 103. Cocoanut. Left, elements of inner fruit coat in surface view: *st* stone cells, *f* bast fibers with *ste* stegmata, *br* brown cells, and *v* vessels. S^1 elements of outer seed coat in tangential section. Right, edible tissues in cross section: S^2 inner layers of seed coat, *E* outer endosperm with oil drops and crystalline fat, and E^2 inner endosperm with crystalloids and network after removal of oil with ether.

Ground cocoanut shells, once a common spice adulterant, consist largely of stone cells with brown contents and stegmata, containing small silicious bodies. Note the large crystalloids, the fat crystals, and the oil drops of the endosperm.

FIG. 104. Olive. Elements in surface view. Fruit coat: *epi* epicarp, *st* stone cell, *ol* oil parenchyma, and *end* endocarp. Seed coat: *aep* outer epiderm and *p* parenchyma with crystals. *E* endosperm. *C* cotyledon with aleurone grains in cross section.

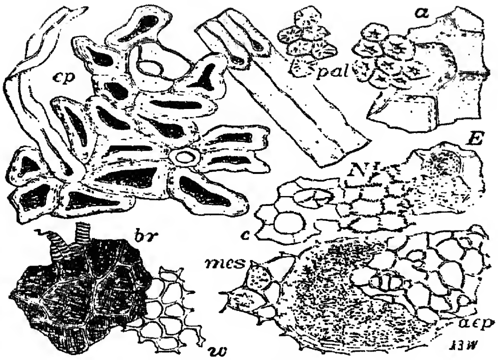
The grotesquely shaped stone cells of the fruit flesh and the colorless stone cells of the olive "pit" occur in olive pomace. The striking seed element is the outer epiderm with large cells and irregularly swollen walls.

FIG. 105. Common Rape. Lower left, seed in cross section: *S* seed coat, *E* endosperm, and *C* cotyledon. Right, elements in surface view: seed coat with *ep* outer epiderm, *sub* subepiderm, *pal* palisade cells, and *pig* pigment cells; *E* endosperm, *C* epiderm of cotyledon with immature stomata.

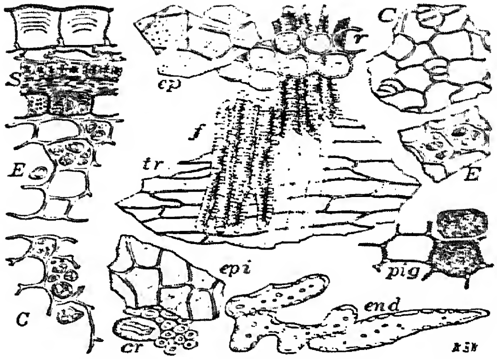
As in all cruciferous seeds (rapes and mustards), oil and aleurone grains are conspicuous. Of value in detecting rape cake or meal are the brown, non-reticulated palisade cells with large central cavities; the epidermal cells are not characteristic.

FIG. 106. Charlock. Upper left, seed in cross section: *S* spermoderm, *E* endosperm, and *C* cotyledon. Right, elements in surface view: seed coat with *ep* outer epiderm, *sub* subepiderm, *pal* palisade cells, *pig* pigment layer; *E* endosperm; *C* epiderm of cotyledon with immature stomata.

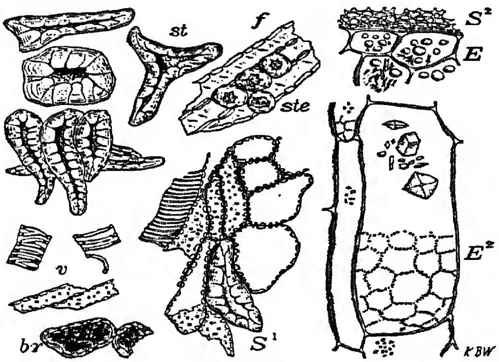
The diagnostic features are the outer epidermal cells filled with cylinders of mucilage, dark palisade cells, and pigment cells.



Cottonseed

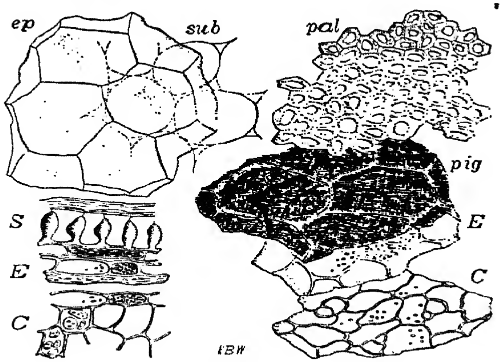


Linseed

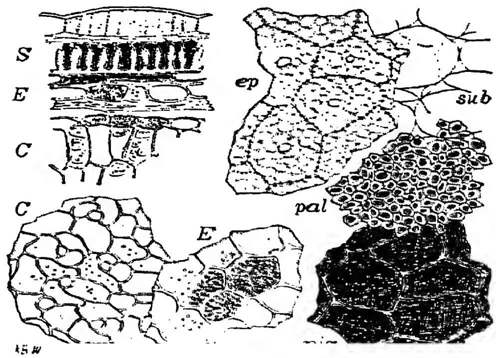


Cocoanut

Olive



Common Rape



Charlock

FIGS. 101–106

Chemical Composition. Oil seeds and nuts, also oil meals (ground oil cakes), are analyzed by the same methods as are employed for cereals and their products and oil fruits by the methods employed for fruits and fruit products, the technique of preparing the sample to meet the greater oil content being varied and the determination of starch being omitted when the iodine test gives a negative result. The oil of air-dry oil seeds is conveniently extracted wholly or in part from a weighed and ground sample in a continuous extractor or by shaking with an oil solvent. The solvent is removed from the residue by evaporation and from the extract by distillation, then both are weighed and separately analyzed.

The pulp and seeds of oil fruits are first separated and weighed, then each is further separated.

Analysis of Oil. See 2, Oils and Fats below.

Analysis of Oil Meal and Extraction Residue. In addition to the usual determination of the six group constituents (Part I, C2), determination may be made of nitrogen distribution (Part I, C4*a*). Isolation methods for the separation of the individual proteins and for the determination of the individual amino acids require more extensive equipment.

The following table gives the average composition of seeds and fruits from which the more common edible oils are prepared.

AVERAGE COMPOSITION OF OIL SEEDS, OIL FRUITS, AND OIL NUTS

	Water	Protein	Fat	Nifext	Fiber	Ash
	%	%	%	%	%	%
Avocado	69.16	2.08	20.10	7.39		1.26
Almond kernel	4.8	21.0	54.9	14.3	3.0	2.0
Black walnut kernel	2.5	30.3	57.8	5.8	1.6	2.0
Brazil nut	4.7	17.4	65.0	5.7	3.9	3.3
Cocoanut kernel	46.31	4.08 *	37.29	7.90	3.39	1.03
Common rape	0.00	21.08	48.55	19.41	6.42	4.54
Cottonseed	0.00	30.29	25.76	36.34	14.37	3.24
English walnut kernel	2.5	16.6	63.4	16.1 †	2.6	1.4
Filbert kernel	3.7	15.6	65.3	13.0		2.4
Hemp	8.75	21.51	30.41	15.89	18.84	4.60
Linseed	6.59	26.55	35.33	28.23		3.30
Olive (pickled):						
Green flesh	58.0	1.1	27.6	11.6		1.7
Ripe flesh	64.7	1.7	25.9	4.3		3.4
Palm kernel	8.40	8.41	48.75	26.87	5.82	1.75
Peanut kernel	5.01	32.06	48.73	9.48	2.44	2.28
Pecan kernel	3.20	11.0	71.99	10.04	2.20	1.57
Pili nut kernel	4.87	13.94	69.58	7.26	0.84	3.51
Pine nut kernel	3.4	14.6	61.9	17.3		2.8
Pistachio nut kernel	4.3	22.8	54.9	14.9		3.0
Poppy seed	8.95	17.86	39.53	22.82	5.46	5.65
Sesame seed	5.61	21.12	46.78	18.63	5.08	6.02
Soy bean	10.80	33.98	16.85	28.89	4.79	4.69
Sunflower kernel	4.00	24.93	50.44	12.83	3.14	4.01

* N × 5.5. † Includes fiber.

2. OILS AND FATS

(Vegetable and Animal)

There is no sharp distinction between fats and true or fatty oils. In the tropics cocoanut oil is liquid, in colder regions it is solid; at room temperature olive oil is liquid, in the refrigerator and in northern winter it is usually solid. Oil without qualification is understood to mean fatty oil as distinguished from mineral oil and essential oil that have little chemical resemblance to fatty oils. Ether extract is of still wider divergence from true oil, as discussed in the introduction to Part I, C2*c*.

Chemically, oils and fats, both vegetable (from seeds and fruits) and animal (from adipose tissue), consist of saponifiable and unsaponifiable constituents. The saponifiable, forming by far the greater part, is a mixture of glycerol triesters (commonly known as glycerides) of saturated acids of the aliphatic fatty series and related unsaturated acids. The unsaponifiable matter consists of a mixture of sterols with less important substances. Nutritionally the glycerides have high calorific value, producing by combustion in the animal organism, like oil in an engine, heat and physical energy, whereas certain of the sterols are of high vitamin D potential. Phosphatides, vitamins A and D, and carotenoids, which also are of great nutritional importance, are miscible with the glycerides and accompany them in the expressed or extracted oils and fats.

Constitution of the Glycerides. Glycerol, the anhydride of which is the essential and characteristic constituent of all fatty molecules, is a trihydric alcohol. The acids combined with glycerol in edible fats and oils belong in three series as follows: (1) saturated acids ($C_nH_{2n}O_2$), (2) unsaturated acids with one double bond ($C_nH_{2n-2}O_2$), and (3) unsaturated acids with two double bonds ($C_nH_{2n-4}O_2$).

By far the most important of these acids are palmitic ($C_{16}H_{32}O_2$), present in most fats and oils, and stearic ($C_{18}H_{36}O_2$), present in fats and most oils, both of the first series, and oleic acid, present in most fats and oils, of the second series. Structural formulas of these three acids, also of linolic acid ($C_{18}H_{32}O_2$) of the third series present in linseed, olive, cottonseed, peanut, sesame, poppy seed, and sunflower seed oils and in cocoa butter, follow:

Palmitic acid

$CH_3-($

Stearic acid

$CH_3-(CH_2)_7-$

Oleic acid

$$CH_3-(CH_2)_7-CH{=}CH-(CH_2)_7-COOH$$

Linolic acid

$$CH_3-(CH_2)_6-(CH{=}CH)_2-(CH_2)_6-COOH$$

Isomers of the unsaturated acids may be assumed to have the double bonds in different positions in the chain.

Because of the double bonds, unsaturated acids are readily oxidized and acted on by the halogens, hence their high iodine and bromine numbers.

Both saturated and unsaturated fatty acids, and their glycerides, are high in carbon content. This is taken into account in the calculation of the fuel value from the analysis, the Rubner factor per gram of fat being 9.3, whereas that for both carbohydates and proteins is only 4.1.

Butter fat contains butyric, caproic, caprylic, capric, lauric, and myristic acid of the first (saturated) series with carbon atoms ranging from 4 to 14 inclusive, in 2 carbon increments, and cocoanut and palm nut oils contain all these acids but butyric. Acids of the second series with carbon atoms as high as 24 occur in peanut and other oils. Fatty acids containing 40 or more carbon atoms have been reported.

Simple and Mixed Glycerides. The three bonds of the anhydride (C_3H_5) of the trihydric alcohol glycerol (glycerin), $C_3H_5(OH)_3$, may be saturated (1) by the anhydride of the same fatty acid forming a simple glyceride, (2) by two of one acid and one of another, or (3) by three different acids; in the last two cases each substance is known as a mixed glyceride. This term should not be confused with the mixtures of glycerides, which may all be of the simple type, in all oils and fats.

Tripalmitin glyceride

Tristearin glyceride

Triolein glyceride

SIMPLE GLYCERIDES

$_5(C_{18}H_{35}O_2)_2(C_{18}H_{33}O_2)$
Distearin monoolein glyceride

Palmitin stearin olein glyceride

MIXED GLYCERIDES

Saponification. By treatment with alkali, as in soap manufacture, and in carrying out certain analytical processes, the anhydrides of the fatty acid or acids are replaced by sodium or potassium, forming respectively hard and soft soap with liberation of glycerol. These soaps in turn, by the action of mineral acids, are decomposed into the free fatty acids with the formation of alkali salts of the liberating acids. Neither soaps nor fatty acids are foods, but one or both are formed in many analytical processes.

Hydrogenation. By means of catalysts, such as nickel and paladium, hydrogen is added to unsaturated oils, thus substituting single bonds for the double and converting liquid oils into solid or semi-solid fats. Treated in this manner, vegetable oils such as cottonseed and cocoanut are brought to a consistency suitable for substitution for butter fat or lard without resorting to the earlier practice of adding beef, lard, or cottonseed stearin. Whale oil thus hardened and rectified is an important food in Europe. Because of the widespread adoption of hydrogenation, physical and chemical values have lost much of their significance in testing fats for their identity or purity.

By practically complete hydrogenation, Mannich and Thiele [1] brought the values of common oils and fats, both vegetable and animal, within the following limits: iodine number 0.0 to 1.2, saponification number 186.2 to 197.7, melting point 62 to 72°, and melting point of the insoluble fatty acids (Hehner) 59 to 71°.

Purpose of Fat Analysis. The large number of oils and fats on the market, some serving for food, others for technical purposes such as soap making and lubrication, are not readily distinguished, if at all, by the senses. Even when their organoleptic properties are well marked, a considerable addition of a characterless foreign oil or fat might escape detection even by the commercial expert.

Considering only oils used for food, at one time much of the so-called olive oil was cottonseed oil or sesame oil, or else consisted in considerable part of these cheaper oils. Today corn oil and peanut oil, sold as such, are formidable rivals of olive oil. Oleomargarine, made from the higher-melting-point fats of beef tallow or lard, was substituted for butter and various cheaper oils or fats or else a mixture such as cottonseed oil and hard animal fat (stearin) was substituted for lard. Today vegetable oils, notably cocoanut oil, more or less hardened, are the chief ingredients of many butter and lard substitutes and in the latter case command a higher price than pure lard. The position of soy oil as an edible oil is at present uncertain.

Fortunately the enforcement of food laws has tended to eliminate frauds, but examination is still essential as a safeguard against

their revival. Inspection of foods from countries where practices are different from those of the home market is particularly important. Fat and oil analysis is of especial value in checking manufacturing processes and in securing uniformity of the products.

The chemist is often called into court in connection with disputes where the evidence as to composition, rancidity, and acidity gained in his laboratory decides the issue.

The quantitative methods for the most part are not analytical in the strict sense as they do not determine the percentages of the individual fatty acids and glycerol, but give numerical expression of physical and chemical properties.

Values of Fats and Oils

Unlike the individual constituents of the carbohydrate, protein, and mineral groups, the individual glycerides or fatty acids are not readily determined as percentages; this necessitates dependence largely on physical values (notably specific gravity, refractive index, and melting point) and conventional chemical values that serve as more or less accurate measures of molecular characteristics, such as molecular weight (saponification number), degree of saturation (iodine number, bromine number) or hydrogenation (acetyl number, hydroxyl number), or the presence of double bonds (diene number, maleic anhydride number).

Certain fatty acids, grouped according to volatility, solubility, liquidity, or solidity, jointly determined as percentages, are also classed as values. The term Hehner number is an equivalent of percentage of insoluble fatty acids.

The early term *constants* has given way to terms less inaccurate or confusing, but nevertheless far from ideal. Lewkowitsch distinguishes between *true constants* or *charac-*

VALUES OF COMMON ANIMAL AND VEGETABLE FATS

	Sp.Gr. 100°/15.5°	Ref. Index 40° C.	Melt. Pt.	Sapon. No.	Iodine No.	Reichert-Meissl No.	Polenske No.	Kirschner No.	Fatty Acids, Titer	Unsaponifiable Matter
									°C.	%
Beef tallow *										
Min.	0.860	1.4566	42	193	35	0.2	0.5	....	42	...
Max.	0.863	1.4586	49	200	45	0.6	0.6	....	45	...
Butter *										
Min.	0.865	1.4527	28	220	22	24.0	1.5	20.0	..	...
Max.	0.870	1.4566	36	241	38	34.0	3.5	28.0	..	...
Lard †										
Min.	0.859	1.4583	36	193	54	0.2	0.4	....	34	0.2
Max.	0.864	1.4609	46	203	70	0.8	0.6	....	42	0.4
Mutton tallow *										
Min.	0.858	1.4586	44	192	32	0.3	...	....	43	...
Max.	0.860	1.4586	49	195	50	0.3	...	....	46	...
Oleo oil *										
Min.	0.860	......	..	198	40	0.3	0.5	....	..	...
Max.	0.863	......	..	202	50	0.4	0.6	....	..	...
Cocoa butter										
Min.	0.950	1.4496	28	193.5	32	0.2	...	....	..	...
Max.	0.976	1.4576	33	...	41	0.8	...	....	..	...
Cocoanut oil *										
Min.	0.863	1.4475	20	246	8	6.0	15.0	1.6	21	0.1
Max.	0.874	1.4495	27	265	10	8.4	20.0	1.9	26	0.3
Palm kernel oil *										
Min.	0.856	1.4494	23	242	10	4.0	6.0	1.0	20	0.2
Max.	0.874	1.4517	30	255	23	8.0	12.0	1.1	26	0.2

* Results on baryta numbers are given under that head.
† Results on baryta numbers and ethyl ester number are given under the respective heads.

Values of Common Edible Oils

	Sp.Gr. 15.5° C.	Ref. Index 25° C.	Hehner No.	Sapon. No.	Iodine No.	Maumené No.	Reichert-Meissl No.	Acetyl No.	Fatty Acids, Titer	Unsaponifiable Matter
									°C.	%
Almond *										
Min.	0.914	1.4680	96.0	183	93	..	0.5	....	..	...
Max.	0.920	1.4710	....	196	105	..	...	....	..	...
Beechnut										
Min.	0.920	......	95.0	191	104	63	...	....	..	...
Max.	0.923	......	96.0	197	121	65	...	....	17	...
Brazil nut										
Min.	0.918	1.4643	....	193	90	50	...	....	31	...
Max.	0.921	1.4681	....	202	107	52	...	....	33	...
Cottonseed †										
Min.	0.922	1.470	....	191	104	50	0.7	21.0	34	0.7
Max.	0.925	1.472	....	195	114	78	0.9	25.0	40	1.7
Eng. walnut										
Min.	0.922	1.4724	....	186	138	90	0.0	....	..	...
Max.	0.927	1.4752	....	198	152	102	...	....	..	...
Hazelnut										
Min.	0.914	1.4665	95.5	187	82	35	0.9	3.2	15	0.5
Max.	0.917	1.4680	....	198	91	37	1.0	...	20	...
Hemp										
Min.	0.925	......	....	190	140	95	...	...	14	1.1
Max.	0.933	......	....	195	166	98	...	...	17	...
Kapok										
Min.	0.9183	1.4591	95.5	181.0	73	95	...	...	..	0.6
Max.	0.9326	1.4657	95.6	196.8	116	..	...	...	..	0.8
Linseed †										
Min.	0.931	1.4782	95.0	190	170	90	0.0	3.9	19	0.5
Max.	0.938	1.4825	95.5	196	202	145	...	...	21	2.0
Madia										
Min.	0.926	......	....	192.8	117	96	...	...	20	...
Max.	0.929	......	....	193.0	121	97	...	...	22	...
Maize										
Min.	0.9213	1.4760	93.0	188.0	110	56	4.0	7.5	13	1.3
Max.	0.9255	1.4768	....	193.4	130	89	5.0	11.5	16	2.9
Niger										
Min.	0.924	1.4741	95.5	188	126	81	0.1	...	25	...
Max.	0.927	1.4767	....	193	134	82	0.7	...	29	...
Olive †										
Min.	0.9155	1.4703	....	189.3	74	38	1.0	10.0	..	0.4
Max.	0.9180	1.4718	....	194.4	95	52	3.0	11.0	..	1.0
Peanut †										
Min.	0.911	1.4707	95.0	186	83	44	0.5	....	23	0.5
Max.	0.926	1.4731	96.0	189	105	51	...	....	32	1.0
Poppy seed †										
Min.	0.9239	1.4730	95.5	189	130	71	0.0	....	15	0.4
Max.	0.9370	1.4750	....	197	158	89	...	....	17	...
Rape										
Min.	0.913	1.4706	94.5	170	93	49	0.0	14.5	6	0.5
Max.	0.917	1.4757	96.5	179	105	65	0.7	14.7	18	1.0
Sesame †										
Min.	0.920	1.4707	95.0	187	103	61	0.5	....	20	0.5
Max.	0.926	1.4725	96.0	193	115	72	2.0	....	24	2.0
Soy bean										
Min.	0.9207	1.4710	95.5	190.1	115	60	...	....	..	0.2
Max.	0.9310	1.4750	....	197.4	145	88	...	....	..	0.2
Sunflower										
Min.	0.920	1.4714	....	188	119	68	...	....	17	0.3
Max.	0.926	1.4735	....	194	140	82	...	....	18	...

* Apricot, cherry, peach, and plum kernel oils fall within practically the same limits.
† Results on baryta numbers are given under that head.

teristics, on the basis of the tendency toward uniform composition of fats and oils from the same seed or fruit, and those widely divergent figures of composition due to method of manipulation or spoilage which he terms variables. Here again there is inconsistency for each natural oil shows a range in its *constants*, usually so wide as to suggest the term *inconstants*. Although the term *number* is the older, *number* and *value* are commonly used interchangeably, causing no little confusion.

In an attempt to clarify the nomenclature partially, in this work the word *values* is used as a general designation for numbers, whether physical or chemical, and in the latter case whether expressed as percentage or in an arbitrary form, and the word *number* is used as a part of a designation for an individual arbitrary term. Thus we class, under *chemical values*, saponification number, iodine number, Hehner number (or per cent of insoluble fatty acids), per cent of unsaponifiable matter or of free fatty acids, individual fatty acids, or groups of fatty acids, etc. Deploring unwarranted innovations, we have made an attempt to stabilize the old.

Tables of Values. Of the two tables above the first is particularly designed to aid in detecting the cheaper oils in the more expensive. The second table, showing values of animal and vegetable fats, includes, in addition to the values given in the first table, those depending on the presence of glycerides of low carbon content which are characteristic of butter fat, cocoanut oil, and palm kernel oil, but are present in very small amount in the animal body fats and vegetable oils of the salad type.

a. Physical Values

Melting Point

Since some of the fats are semi-solid at living room temperatures, there is a choice of determining the melting point, after cooling to strict solidification, or the solidifying point, after melting to a clear liquid. The former practice seems preferable. The two values are not identical.

Wiley Disk Method.[2] This method, devised by Wiley for the examination of butter fat,[3] is well adapted for other fats.

Apparatus. *Melting Point Assembly.* A tall beaker, containing water, rests on a lamp-stand ring in which is immersed a test tube 3.5 cm. in diameter and 30 cm. high, containing ethanol diluted to the specific gravity of the fat. An ordinary thermometer is suspended in the beaker and one accurate to 0.1° in the tube. A bent tube connected with a blowing-bulb serves to stir the water in the beaker.

Process. *Preparation of Fat Disks.* Form disks 1.0 to 1.5 cm. in diameter, weighing about 0.2 g., by allowing the melted and filtered fat to drop 15 to 20 cm. from a tube onto a smooth piece of floating ice. Press the ice under water and remove the floating fat with a spatula.

Treatment of Fat Disks. Boil separately for 10 minutes *ethanol* and distilled water. Without cooling, pour the water into the test tube until half full, then nearly fill with the hot *ethanol* carefully poured down the sides, avoiding mixing. Place the tube in the beaker of water cooled by ice. When the temperature is reduced to below 10°, let fall a disk of fat from the spatula into the liquid. To secure an even temperature, from time to time gently stir with the thermometer the liquid in the region where the disk settles.

Heat slowly the water in the beaker, stirring with the blown-in air current. When the temperature is about 6° below the melting point and the disk begins to shrivel and slowly rolls up into an irregular mass, adjust the accurate thermometer so as to show the temperature about the fat and gently rotate. Regulate the rise so that the last 2 degrees

require about 10 minutes. Read when the fat assumes a definite spherical form.

Capillary Tube Method. The capillary tube is the usual form except that it is not closed at one end. Draw a portion of the fat into the tube, either seal or preferably leave open as sealing in the flame burns the fat, and cool in the refrigerator overnight. Attach to the thermometer so that the fat and bulb are at the same level. Heat slowly (0.5° per minute) and note the temperature at which the fat liquifies.

Melting Point Number

Bömer and Limprich Method.[4] The method is based on the preponderance of α-palmitodistearin in lard and of β-palmitodistearin in beef and mutton tallow, the melting point of the two glycerides being 68.5 and 63.3° and of their fatty acids 63.3 and 63.2° respectively, all corrected.

Process. *Crystallization.* Allow a solution of 50 g. of the clear fat in 50 ml. of *ether* to crystallize for 1 hour at 15° in a 150-ml. beaker covered with a watch glass, with frequent stirring. If the yield of crystals is small, employ a lower temperature (5 to 10°) and allow to stand longer or use *anhydrous acetone* or *ethanol-ether mixture* (3 + 1 or 4 + 1) instead of ether. Filter with suction, press dry with a watch glass, dissolve the crystals in the same amount of *solvent* as before, recrystallize, and filter, repeating until the crystals melt at over 61°, which is usually after the second crystallization.

Fatty Acids Preparation. Grind 0.1 to 0.2 g. of the glycerides thus obtained in a small mortar and saponify about half by heating in a small beaker with 10 ml. of *0.5 N ethanolic potassium hydroxide solution* for 5 to 10 minutes on an asbestos plate. Transfer to a separatory funnel with 100 ml. of water, add to the soap solution (which must be clear) 2 to 3 ml. of *25% hydrochloric acid*, and shake out with 25 ml. of *ether*. Wash the ether solution with two 25-ml. portions of water, filter through a dry paper, evaporate the ether, dry the fatty acids 30 to 60 minutes in a boiling water oven, cool, and powder.

Melting Point. Determine at the same time the melting point of the fatty acids and of the glycerides compacted into columns 2 to 3 mm. high in U-shaped capillary tubes of uniform diameter (¾ mm.) attached to opposite sides of the thermometer by heating in water so as to cause a rise in temperature of 1.5 to 2° per minute and reading when transparent.

Calculation. Calculate D by subtracting from the melting point of the glycerides (G) the melting point of the fatty acids (A).

If a value for $G + 2D$ less than 71 is obtained with lard, tallow or a hydrogenated vegetable oil is indicated. This latter may be detected by the phytosterol acetate test carried out on the filtrate from the first crop of glyceride crystals after evaporating the ether and saponifying.

Examples. The following values for $G + 2D$ were obtained by Bömer and Limprich:

Lard (16 samples)	73.4–78.1
Beef tallow (2 samples)	62.8–67.0
Mutton tallow	63.9–66.0

Specific Gravity

Specific gravity may be determined by means of one of the three pieces of apparatus described in the Introduction. The hydrometer and the Westphal balance have the advantage of convenience and ease of cleaning, the pycnometer of accuracy. The temperature of 15.5° C. (60° F.), regarded as the average temperature of laboratories, has long been standard. It is true that modern laboratories are kept at a higher temperature, but it is still true that tap water in the northern states commonly is no warmer than 15.5°. The movement in the United States to adopt 25° as the temperature for oils necessitates recalculation for comparison

with the vast amount of data in such works as Lewkowitsch, Allen, Mitchell, and Jamieson. Changes in standards and nomenclature have wrought confusion in chemistry as well as botany.

In calculating to 15° results obtained at other temperatures, add 0.00064 for each degree above 15° and subtract the same for each degree below 15°.

Refraction

Butyro-Refractometer Method. In addition to the Abbé refractometer, which gives a wide range of refractive indices, Zeiss also makes a butyro-refractometer, with a narrower range and an arbitrary scale graduated from 0 to 100 corresponding to refractive indices 1.422 to 1.489, which, although specially adapted to the examination of butter and its substitutes, may also be used for most edible oils and fats. The instrument has the advantage over the Abbé instrument of simpler construction, greater convenience in operation, less cumbersome scale, and various colorings of the critical line due to high (blue) or low (red) dispersion, which are characteristic of certain oils and fats. However, this coloring obscures the fringes obtained with certain oils and prevents an accurate reading, although the interference is reduced by using a sodium light.

The prisms in both instruments are the same. Since the scale of the butyro-refractometer is within the tube, it may be read directly without special manipulation. In other respects the manipulation is like that of the Abbé instrument.

The readings of solid fats, such as lard and butter, may be corrected for temperature by adding 0.55 for each degree above or subtracting 0.55 for each degree below the desired temperature. In the methods of the A.O.A.C., 0.58 is given as the correction for

Readings of the Zeiss Butyro-Refractometer and Corresponding Refractive Indices

Reading	Refractive Index	Reading	Refractive Index	Reading	Refractive Index	Reading	Refractive Index	Reading	Refractive Index
40.0	1.4524	48.0	1.4579	56.0	1.4633	64.0	1.4685	72.0	1.4735
40.5	.4527	48.5	.4583	56.5	.4636	64.5	.4688	72.5	.4738
41.0	.4531	49.0	.4586	57.0	.4639	65.0	.4691	73.0	.4741
41.5	.4534	49.5	.4590	57.5	.4642	65.5	.4694	73.5	.4744
42.0	.4538	50.0	.4593	58.0	.4646	66.0	.4697	74.0	.4747
42.5	.4541	50.5	.4596	58.5	.4649	66.5	.4700	74.5	.4750
43.0	.4545	51.0	.4600	59.0	.4652	67.0	.4704	75.0	.4753
43.5	.4548	51.5	.4603	59.5	.4656	67.5	.4707	75.5	.4756
44.0	.4552	52.0	.4606	60.0	.4659	68.0	.4710	76.0	.4759
44.5	.4555	52.5	.4609	60.5	.4662	68.5	.4713	76.5	.4762
45.0	.4558	53.0	.4613	61.0	.4665	69.0	.4717	77.0	.4765
45.5	.4562	53.5	.4616	61.5	.4669	69.5	.4720	77.5	.4768
46.0	.4565	54.0	.4619	62.0	.4672	70.0	.4723	78.0	.4771
46.5	.4569	54.5	.4623	62.5	.4675	70.5	.4726	78.5	.4774
47.0	.4572	55.0	.4626	63.0	.4678	71.0	.4729	79.0	.4777
47.5	.4576	55.5	.4629	63.5	.4681	71.5	.4732	79.5	.4780

oils. For corn (maize) oil, 0.62 is more accurate. The most commonly used temperatures are 25° for oils and 40° for fats.

Abbé Refractometer Method. Determine the index of refraction in the usual manner. Correct for temperature by adding 0.000365 for each degree above or subtracting that figure for each degree below the desired temperature, as recommended by Tolman.[5] Conversion of the readings on the butyro-refractometer scale into refractive indices and vice versa may be made by means of the table above prepared by Winton and

Heat-Rise Values

Several methods depending on rise in heat with certain reagents are rough measures of the degree of unsaturation.

Tests based on the rise in heat on mixing an oil with sulfuric acid, bromine, or some other reagent that reacts more actively with glycerides of unsaturated oils than with those of saturated oils, although not of strict scientific accuracy, are of some value as a guide.

Maumené Sulfuric Acid Method.[7] The determination of the Maumené number was formerly more highly regarded than at present and was the subject of numerous investigations. It is at least worthy of description. Each worker should make observations with oils of known origin and establish his own standard.

Process. Place 50 g. of the oil in a tube or beaker, provided with an asbestos, felt, or vacuum jacket, and observe the temperature as shown by a standard thermometer. Add from a slowly delivering pipet 10 ml. of *sulfuric acid* of the same temperature as the oil. During the addition of the acid, stir with the thermometer, to which is attached, to aid in mixing, a piece of sheet platinum by means of a loop formed by two slits, then suspend it in the mixture and observe the highest temperature attained.

The difference between the initial and highest temperature is the Maumené number.

Mix an oil, such as fish or linseed, yielding a high value, with an equal weight of olive oil and multiply the difference between the readings on the mixture and the olive oil by 2.

Hehner and Mitchell Bromine Method.[8] This method is stated to have advantages over the Maumené method.

Process. Dissolve 1 g. of the oil in 10 ml. of *chloroform* contained in a jacketed test tube. Add 1 ml. of *bromine* from a delicate pipet provided with a mouthpiece containing *quicklime*, and at each end an asbestos plug, both oil and bromine being of the same initial temperature. Note the rise in heat on a thermometer graduated in fifths of a degree.

Calculation. Proceed as directed under the Maumené Method.

Polenske Difference Number

The difference number measures the stretch between the melting point and the solidifying point.

As a means of distinguishing different animal fats, Polenske [9] obtained the difference between the melting point, as determined in the capillary tube, and the solidifying point, as represented by the temperature at which turbidity appears in the melted fat. Uniformity of apparatus and manipulation are essential.

Examples. Polenske obtained the following results: butter fat 11.8 to 14.3°, lard 19.5 to 20.5°, and tallow 12.8 to 14.2°.

Notes. Fischer and Alpers [10] and Laband [11] found the figures useful in certain cases, but inadequate in the examination of mixtures.

Fischer and Wewerinke [12] prefer to calculate the value $3D - S$ (in which D is the usual Polenske difference number and S is the solidifying point), which for lard is usually over 30 and practically never below 28.

Grau [13] reports in German lard difference number and per cent of solid fatty acids respectively as follows: back fat lard, sample *A* 10.7 and 38.2, sample *B* 13.3 and 38.6; belly fat lard, 18.3 and 48.2.

Titer Test

The solidifying point of the fatty acids of a fat is known as the titer.

Modified Dalican Method. The original method credited to Dalican, an early oil chemist, has been modified by Finkener in Germany, Wolfbauer in Austria, and Wesson in the United States, without material change in the ethanol-alkali saponification. More recently the Committee on Analysis of Commercial Fats and Oils of the American Chemical Society [14] adopted glycerol-alkali saponification and other details essentially as follows.

Apparatus. *Standard Thermometers.* Two forms are specified. The one for low titer is graduated from 10 to 65°, the other for high titer (above 55°) from 30 to 85°, both by tenths of degrees. The 10° mark is 3 to 4 cm. above the bulb, the total length being 37 cm. The bulb of moderately thin glass is about 3 cm. long and 6 mm. in diameter; the stem is also 6 mm. in diameter. Jena glass annealed at 450° for 75 hours and certification by the U. S. Bureau of Standards are required.

Reagent. *Glycerol-Alkali.* Dissolve 250 g. of KOH in 1 liter of dynamite glycerol with the aid of heat.

Process. To 75 ml. of *glycerol-alkali* heated to 150°, add 50 g. of the oil or melted fat, stir well, continuing the heating until the mixture is homogeneous and avoiding a temperature above 150°. Cool somewhat, add cautiously 50 ml. of *1 + 3 sulfuric acid*, then hot water, and heat until the fatty acids separate as a clear layer.

Transfer to a separatory funnel, remove the acid solution, and wash the fatty acids with hot water until the washings are neutral. Pour off and filter the fatty acids, dry rapidly at 150° with stirring, cool somewhat, and transfer to a titer tube, 2.5 x 10 mm., fitted through a perforated cork into a widemouth bottle. Suspend a thermometer in the fatty acids and use it as a stirrer, rotating at about 100 r.p.m. until the temperature remains constant for 30 seconds. Immediately discontinue the stirring, allow the thermometer to hang in the center of the fatty acids, and record the highest temperature attained as the titer.

For all fats with a titer above 30°, employ a temperature of about 20°; for all other fats, employ a temperature 10° below the titer.

Examples. See tables in the early part of the chapter.

Note. The above procedure is one of the two A.O.A.C. Official Methods, the other being the alcoholic or aqueous sodium hydroxide method, with explicit specifications for a standard thermometer graduated from −2 to 62° in 0.2°.

b. Chemical Values

Saponification Number

The Köttstorfer or saponification number is the number of milligrams of potassium hydroxide necessary to saponify completely 1 g. of the oil or fat. It is a measure of the average molecular weight of the mixed glycerides constituting a given fat or oil. Although the range is not nearly so great as that of the iodine number, the saponification number is an important constant, particularly in distinguishing rape, mustard, and other cruciferous oils from most of the other edible oils, in identifying cocoanut oil, and in an exhaustive examination of butter and substitutes.

For many years a method for the determination of the saponification number, solu-

ble fatty acids, and insoluble fatty acids in one weighed portion has been official with the A.O.A.C.[15] as follows.

Köttstorfer Volumetric Method.[16] Apparatus. *Saponification Assembly* (Fig. 107).

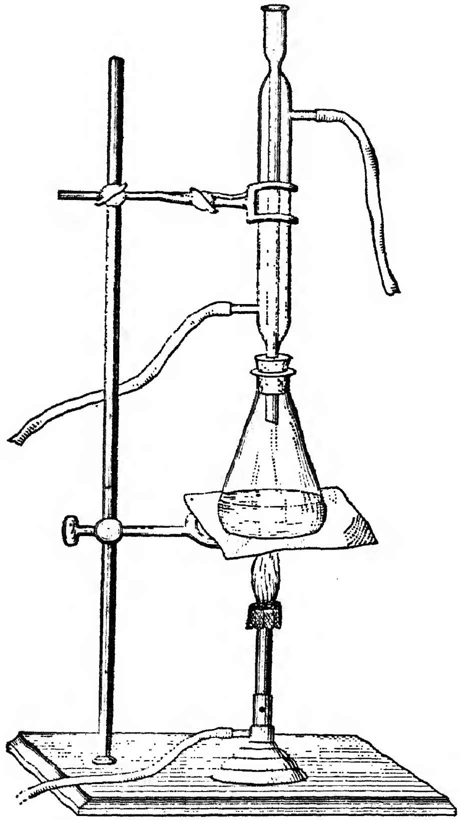


Fig. 107. Saponification Assembly.

Reagents. *Ethanolic Potassium Hydroxide Solution.* Dissolve 40 g. of KOH in 1 liter of ethanol, previously purified by standing some days with KOH and distillation. The solution is approximately 0.7 *N*.

Standard 0.5 N Hydrochloric Acid. Prepare in the usual manner and standardize.

Process. *Saponification.* Weigh accurately an Erlenmeyer flask of about 200 ml. capacity, introduce 5 g. of the oil or melted fat (1.5 to 2 g. of butter or cocoanut oil), and weigh again. Add from a pipet exactly 50 ml. of the *ethanolic potassium hydroxide solution,* connect with a reflux condenser, and boil gently, by heating over a piece of asbestos paper, for 30 minutes.

Titration. Cool, add a few drops of *phenolphthalein indicator,* and titrate the excess of alkali with *standard 0.5 N hydrochloric acid.* Reserve the neutralized liquid for the determination of soluble and insoluble fatty acids below.

Blank. Conduct blank determinations in exactly the same manner. It is immaterial whether the quantity of potash solution discharged from the pipet is exactly 50 ml., but of great importance that the quantity be exactly the same in the blank as in the actual analysis. Care therefore must be taken to use the same pipet and allow it always to drain for exactly the same length of time.

Calculation. Subtract from the average number of milliliters of 0.5 *N* acid, obtained in closely agreeing duplicate blank determinations, the number obtained in each actual analysis, multiply the result by 28.06 (the number of milligrams of potassium hydroxide corresponding to each milliliter of 0.5 *N* acid), and divide the product by the weight of material employed.

Examples. See the table under Values of Fats and Oils above.

Note. Details of the further procedure for the determination of soluble and insoluble fatty acids on the same portion are given in subsequent sections.

Neutralization Number

The number of milligrams of potassium hydroxide required to neutralize 1 g. of the fatty acids is known as the neutralization number.

As some fatty acids contain unsaponified fat, the saponification number, which includes the neutralization number, may be determined and calculated in the same manner as for oils and fats. If no unsaponified fat is present, the two numbers are practically identical.

Mean Molecular Weight

The following formula for the calculation of the mean molecular weight (M) from the neutralization number (N) is applicable when the fatty acids are free from fat and other impurities:

$$M = \frac{56,100}{N}$$

The calculation may also be made directly from the weight of the fatty acids (W) and the number of milliliters obtained in titration, as directed under Neutralization Number above.

Reichert-Meissl Number

The Reichert-Meissl number is the number of milliliters of 0.1 N alkali required to neutralize the liquid volatile fatty acids of 5 g. of the oil or fat.

Reichert-Meissl Volumetric Method. This process is used chiefly in distinguishing oleomargarine from butter. It depends on the presence in butter fat of a considerable amount of glycerides of volatile soluble acids of the fatty series with low carbon content, chiefly butyric acid ($C_4H_8O_2$), together with smaller amounts of caproic acid ($C_6H_{12}O_2$), caprylic acid ($C_8H_{16}O_2$), and capric acid ($C_{10}H_{20}O_2$), whereas the animal fats and most vegetable fats contain little more than traces, and cocoanut and palm kernel oils contain intermediate amounts consisting chiefly of caproic, caprylic, and capric acids. These acids, of which the Reichert-Meissl number is a measure, are not only volatile on distillation with steam, but are also quite soluble in water.

Reichert was the first to make use of these facts in a method for detecting oleomargarine. The process was later improved by Meissl and later still by Leffmann and Beam, who employed for the saponification a mixture of glycerol and aqueous sodium hydroxide solution instead of ethanolic sodium hydroxide solution.

Polenske [17] in 1904 further modified the process so as to determine the volatile acids, insoluble, as well as those soluble, in water, thus differentiating cocoanut oil, which contains a considerable amount of glycerides of these insoluble volatile acids, notably lauric acid ($C_{12}H_{24}O_2$) and myristic acid ($C_{14}H_{28}O_2$), from butter. The following figures illustrate the value of both determinations.

	Reichert-Meissl No.	Polenske No.
Butter fat, 31 samples (Polenske)	23.3–30.1	1.5– 3.0
Cocoanut oil, 4 samples (Polenske)	6.8– 7.7	16.8–17.8
Oleomargarine fat (Arnold)	0.5	0.53
Lard (Arnold)	0.35	0.5
Tallow (Arnold)	0.55	0.56

Butter contains volatile acids with 4 to 14 atoms of carbon and cocoanut oil contains acids with 6 to 14 atoms, both inclusive. It should be further noted that butter fat contains a considerable amount of the acid with 4 atoms of carbon (butyric), which is not found in cocoanut oil, whereas cocoanut oil contains considerable amounts of acids with 12 and 14 atoms (lauric and myristic), which occur only in small quantities in butter fat. Since the solubility decreases as the number

of carbon atoms increases, it is obvious why butter fat gives a high Reichert-Meissl number and cocoanut oil a high Polenske number.

Leffmann and Beam Glycerol Modification.[18] Since the volatility and solubility of

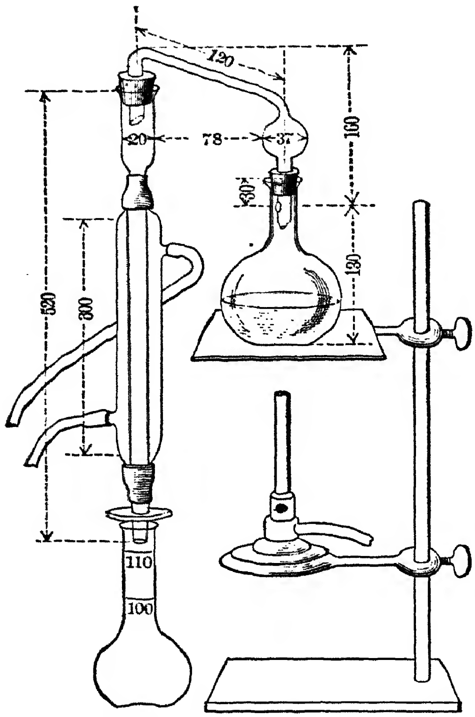


FIG. 108. Volatile Fatty Acids Distillation Apparatus.

the fatty acids vary with the conditions, it is necessary to adhere closely to a conventional method in order to obtain concordant results. The following description in all essential details conforms to the A.O.A.C. method.

REAGENTS. *Glycerol-Sodium Hydroxide Solution.* Prepare a solution of 50 g. of dry NaOH in 50 ml. of boiled water. Allow to settle and mix 20 ml. of the clear solution with 180 ml. of concentrated glycerol.

Sulfuric Acid, 20%. To 100 ml. of water add 25 ml. of H_2SO_4.

Standard Potassium or Sodium Hydroxide Solution, 0.1 *N*.

PROCESS. *Saponification.* Weigh accurately a 300-ml. Jena flask, introduce as much of the melted fat as will be delivered by a clean, dry 5-ml. pipet and enough more to bring the weight up to exactly 5 g. Allow to cool and weigh accurately flask and fat. Add 20 ml. of *glycerol-sodium hydroxide solution* and heat cautiously on a piece of asbestos paper until the fat is saponified; this requires about 5 minutes and is indicated by the clearing up of the boiling liquid. While still hot, add very cautiously, at first drop by drop to prevent foaming, 135 ml. of boiled water and shake until the soap is dissolved. The solution should be perfectly clear and nearly colorless. Rancid or oxidized fats that yield a brown soap should not be examined.

Distillation (Fig. 108). To the soap solution, add 6 ml. of *1 + 4 sulfuric acid* and about 0.5 g. of *granulated pumice stone* with grains 1 mm. in diameter, then connect with a condenser and distil into a 110-ml. volumetric flask at a rate sufficient to give a distillate of 110 ml. in about 30 minutes, using a stream of water that will cool the condensed liquid to about 20°. Cool in water of about 15°, make up to the mark, mix by inverting the flask 4 or 5 times, and filter through a 9 cm. dry paper.

Titration. Without further addition of water, titrate with *standard 0.1 N alkali* 100 ml. of the filtrate, using a few drops of *phenolphthalein indicator.* Correct for the reading by deducting the reading obtained in a blank determination.

CALCULATION. Multiply the corrected number of milliliters of standard alkali required by 1.1, thus obtaining the Reichert-Meissl number.

EXAMPLES. See table under Values of Fats and Oils above.

Polenske Number

The Polenske number is the number of milliliters of 0.1 N alkali required to neutralize the solid volatile fatty acids of 5 g. of the oil or fat.

For this determination, wash the condenser tube, flask, and filter, after obtaining the Reichert-Meissl number as described above, with three 15-ml. portions of water and dissolve the insoluble volatile acids by the same treatment, using 15-ml. portions of *neutral 90% ethanol.* Finally titrate the united ethanolic washings as in the determination of the soluble acids.

Calculation. Do not multiply by 1.1 since the whole amount of the solid volatile acids is used for the titration.

Examples. See table under Values of Fats and Oils above.

Kirschner Number

Kirschner Silver Sulfate Volumetric Method.[19] *The portion of the soluble acids, consisting chiefly of butyric, that is not precipitated by silver sulfate is measured by the Kirschner number.* It is determined in the distillate obtained by the Reichert-Meissl process and is calculated in a manner analogous to that employed in the determination of the Reichert-Meissl and Polenske numbers.

Bolton and Revis Modification.[20] Process. *Silver Precipitation.* After titration to a faint pink color with *standard alkali solution* of 100 ml. of the filtered distillate in the Reichert-Meissl determination, without dilution add 0.3 g. of *powdered silver sulfate* and shake thoroughly at frequent intervals for 1 hour. Filter on a dry paper and pipet 100 ml. of the filtrate into a 300-ml. flask. Add 35 ml. of water, 10 ml. of *1 + 40 sulfuric acid*, and a piece of *aluminum wire* or a few pieces of *pumice stone.*

Distillation and Titration. Distil 110 ml. and titrate 100 ml. as for the Reichert-Meissl number. Make a blank determination and correct the reading.

Calculation. Calculate the Kirschner number (K) by the following formula:

$$K = \frac{11}{10} \times \frac{11}{10} \times \frac{k(100 + r)}{100}$$
$$= 0.0121k(100 + r)$$

in which k is the corrected number of milliliters of 0.1 N alkali added in the Kirschner titration and r is the corrected number of milliliters added in the Reichert-Meissl titration, both corrected for the amount found in a blank analysis.

Example. See table in Part II, G7.

Butyric Acid Number; Total Volatile Acids Number; Residue Number; Lauric Acid Number

The butyric acid number is the value in terms of 0.01 N alkali per 0.5 g. of fat of the volatile acids distilled from the acidulated saponified fat after salting out with alkali sulfate and treatment with a saturated solution of caprylic acid from cocoanut oil. The *total volatile acids number* in the same terms is determined by a similar process but omitting the addition of caprylic acid and substituting magnesium sulfate for alkali sulfate. The *residue number* is the total volatile acids number less the butyric acid number. The *lauric acid number* is derived from a formula given below.

Grossfeld Semi-Micro Volumetric Method.[21] Reagents. *Potassium Hydroxide Solution*, ethanolic. Dilute 40 ml. of a concentrated aqueous solution (sp.gr. 1.5) with 90% ethanol.

Kieselguhr, purified. Boil with 1 + 1 hydrochloric acid, wash free of acid, and dry at 100°.

Cocoanut Oil Soap Solution. Boil gently with shaking 100 g. of cocoanut oil, 100 ml. of glycerol, and 40 ml. of potassium hydroxide solution. Allow the clear solution to stand, then dilute to 1 liter.

A. Butyric Acid Number. Process. *Saponification.* Weigh 0.5 to 0.55 g. of the

sample into the 50-ml. flask of a reflux assembly with a 20-cm. condenser and a hot-plate heater. Add a pinch of coarsely ground *pumice stone* and 5 ml. of *ethanolic potassium hydroxide solution*, then heat the mixture to boiling and boil gently for 5 minutes after the fat has dissolved. Disconnect the flask from the condenser, add 1 ml. of *glycerol* (sp.gr. 1.23), and boil without the condenser until most of the ethanol has been driven off as indicated by the beginning of foaming. In order to remove the last traces of ethanol, heat in a boiling water oven in an inclined position for about 1 hour.

Fatty Acids Liberation. Immediately after removing from the oven, add 15 ml. of *saturated potassium sulfate solution* (sp.gr. 1.08), cool to 20°, then add with shaking 0.5 ml. of *1 + 3 sulfuric acid*, 1 ml. of *cocoanut oil soap solution*, and about 0.1 g. of purified *kieselguhr*. Filter through a pleated 10-cm. paper into a graduated tube of the Beckel type until the filtrate reaches the 12.5-ml. graduation in the constricted portion, pressing the precipitate with the rounded bottom of a small test tube if necessary to obtain the full amount of liquid.

Volatile Fatty Acids Distillation. Transfer the filtrate to a 100-ml. flask, rinsing the tube with 5 ml. of water (indicated by a mark on the body of the tube), and distil until 11 ml. are collected in the receiver.

Titration. To the distillate, add a few drops of *1% phenolphthalein solution* and titrate with *standard 0.01 N sodium hydroxide solution* until a faint red color appears that persists on stirring.

Blank. Make a control determination, using cocoa butter (not cocoanut oil).

CALCULATION. To obtain the semi-micro butyric acid number (B), subtract the reading of the control from that of the actual analysis and multiply by the factor (F) obtained as follows:

$$F = \frac{50}{\quad} \times \frac{17.5}{\quad} \qquad 70$$

in which C is the weight of the charge. Grossfeld's table gives the factor directly.

B. Total Volatile Acids Number. PROCESS. *Saponification.* Proceed as directed under Saponification above, substituting a 100-ml. for the 50-ml. flask.

Fatty Acids Liberation. Dissolve the warm soap solution in 50 ml. of water, add with shaking 25 ml. of *1.5% magnesium sulfate solution*, and allow to stand overnight. Filter through a dry pleated 15-cm. paper into a 100-ml. graduated cylinder. If necessary to obtain 50 ml. of filtrate, squeeze the precipitate with the rounded bottom of a small test tube.

Distillation. Pipet the 50-ml. filtrate into a 100-ml. flask, add about 0.1 g. of *coarsely powdered pumice* and 1 ml. of *sirupy phosphoric acid solution*, and distil into a measuring cylinder until filled to the 40-ml. mark.

Titration. Transfer the distillate to a beaker, using 10 ml. of *neutralized 0.02% phenolphthalein solution in ethanol* to rinse the condenser and receiver, and titrate with *standard 0.01 N sodium hydroxide solution.*

Blank. Carry along a blank determination, in the same manner and with the same reagents as in the actual analysis, omitting the fat but using in the magnesium precipitation 0.2 to 0.3 g. of *purified kieselguhr.*

CALCULATION. Correct the number of milliliters used in the actual analysis by subtracting the number required in the titration of the blank and obtain the total volatile acids number (T) by multiplying the difference by the factor (F') derived by the following formula:

$$F' = \frac{76.0}{50} \times \frac{500}{C'} = \frac{760}{C'}$$

in which C' is the weight of the charge in grams. The calculation of the factor is avoided by consulting Grossfeld's table.

The amount of butter fat (F) and of cocoanut fat (F') may be calculated by the following formulas respectively:

$$F = 5.12B - 0.12R$$

and

$$F' = 2.77R - 2.63B$$

in which B and R are the butyric acid number and residue number respectively.

C. Residue Number. Subtract B from T.

D. Lauric Acid Number. The following formula gives the lauric acid number (L):

$$L = 3.3(S - 0.7B - 0.6R - 197)$$

in which B and R are the butyric acid and residue numbers, S is the saponification number, and 197 is the saponification number of the basic fat (lard, cacao butter, etc.).

EXAMPLES. Grossfeld reports the following figures for total and residue acid numbers respectively: cocoanut oil 38.0 and 37.1; lard 0.3 and 0.2; cacao butter 0.0 and 0.0; butter 39.7 and 193. In a later paper Grossfeld, Schweizer, and Damm [22] give the following averages for 245 samples of butter fat: butyric acid number 20.0; total volatile acids number 34.8; and residue number 14.8.

Grossfeld Simplified Modification.[23] PROCESS. *Saponification.* Measure from a 1-ml. graduated pipet 0.6 ml. of the melted fat into a 50-ml. flask, add 5 ml. of *4% ethanolic potassium hydroxide solution,* heat on a hot plate (iron, aluminum, or asbestos) to boiling, and boil gently until the fat has dissolved. For each fat sample employ a separate pipet. Add to each test from a 10-ml. graduated pipet with 1-ml. graduations, and not too narrow delivery, 1 ml. of *glycerol* and boil the soap solution until foaming begins. Remove the ethanol by drying at 100° 10 to 15 minutes, inclining the flask.

Volatile Acids Distillation. Dissolve the still hot soap in 15 ml. of *saturated potassium sulfate solution* and add with shaking 0.5 ml. of *1 + 3 sulfuric acid,* 1 ml. of *cocoanut soap solution,* and a pinch of *purified kieselguhr.* Filter on a 10-cm. pleated paper into a Beckel tube until the 12.5-ml. mark is reached, transfer the filtrate into a 100-ml. flask, rinse with 5 ml. of water, and distil 11 ml.

Titration. Using *phenolphthalein indicator,* titrate with *0.01 N sodium hydroxide solution.* Deduct the number of milliliters obtained in a blank on 0.6 ml. of cacao butter.

Interpretation. If the result (simplified butyric acid number) is over 11 (= total acid number 17), it is usually safe to conclude that the sample is butter fat; if below 11, calculate the approximate butter fat content (per cent) by multiplying by 7.

Check. In case of a dispute, it is desirable to make an accurate determination of butyric acid number (B). If $B \times 5$ agrees with the result obtained as directed above, further check is unnecessary.

CALCULATION. Determine the total volatile fatty acids number (T) by the Grossfeld semi-micro method, then obtain the residue number (R) by the formula

$$R = T - B$$

also the per cent of butter fat (M) and of cocoanut oil (C) by the formulas

$$M = 5.12B - 0.12R$$
$$C = 2.77R - 2.63B$$

BARYTA NUMBERS

The soluble and insoluble baryta numbers indicate roughly the content of volatile insoluble fatty acids with 6 to 13 carbons in the molecule.

The method as proposed by Koenig and Hart,[24] even with the modifications of Kreis and Baldin,[25] and Laves,[26] was found to be unsatisfactory. The following method, devised at the Hygienic Institute of Leipsig University, however, has come into use in England and Germany.

Avé-Lallemont Gravimetric Method.[27] Bolton and Revis [28] have demonstrated that the method serves the double purpose of detecting cocoanut oil and lard compounds, the influence of the two together being additive.

REAGENT. *Barium Chloride Solution,* 2.5%. Standardize accurately.

PROCESS. *Saponification.* Saponify 1.9 to 2.0 g. of the sample with 25 ml. of approximately *50% ethanolic alkali* for 30 minutes, as in the determination of the saponification number by the Köttstorfer method. To the warm soap solution, add 3 to 4 drops of *2% phenolphthalein solution* and neutralize with *0.5 N hydrochloric acid.* Dealcoholize as completely as possible by heating on a boiling water bath in a current of air; then add to the hard soap 10 ml. of water and continue the heating and aeration until the mass again hardens.

Baryta Soap Precipitation. Dissolve in air-free boiling water and transfer to a 250-ml. volumetric flask, thus bringing the total volume up to 150 to 180 ml. Heat again for 5 minutes on the water bath and add from a pipet with gentle shaking 50 ml. of *2.5% barium chloride solution.* Continue the heating for about 15 minutes or until the insoluble baryta soaps coagulate. Cool, fill to the mark, shake, filter through a dry paper (returning the first portion that runs through to the paper), and remove 200 ml. of the filtrate to a beaker.

Barium Sulfate Precipitation. Add 1 ml. of *hydrochloric acid*, dilute to 350 ml., heat to boiling, add drop by drop an excess of *dilute sulfuric acid*, and allow to stand some hours. Collect the barium sulfate on a Gooch crucible, wash, and ignite at dull redness in the usual manner.

CALCULATION. Calculate the *insoluble baryta number* (I) by the following formula:

$$I = \frac{A - (1.25 \times 0.657 \times B)}{G}$$

in which A is the milligrams of barium oxide equivalent to 50 ml. of the barium chloride solution, 0.657 is the conversion factor for barium sulfate to oxide, B is the milligrams of barium sulfate obtained in the actual analysis, and G is the grams of fat used in the analysis.

Calculate also the *total baryta number* (T)

BARYTA NUMBERS OF OILS AND FATS

Fat or Oil	Total Baryta No. T	Insoluble Baryta No. I	Soluble Baryta No. S	Difference No. $D = I - (200 + S)$
Butter fat	297.3 *–329.6	242.6–254.8 †	50.6 ‡–76.7	−24.8– −0.7 §
Lard	265.0–267.7	253.5–259.2	6.3–12.2	+41.5–+52.9
Beef tallow	267.1–270.3	261.4–264.9	2.2– 7.4	+45.0–+62.7
Mutton tallow	266.8	256.0	10.8	+45.2
Oleo oil	263.3–266.6	254.5–257.0	8.7– 9.6	+45.5–+47.4
Cocoanut oil	351.8–354.1	296.5–299.2	54.1–57.6	+38.9–+45.1
Palm kernel oil	336.6	303.7	32.9	+70.8
Cottonseed oil	263.5–265.8	255.2–256.9	6.6–10.6	+44.6–+50.3
Peanut oil	256.7–258.0	242.2–255.0	1.7–15.8	+26.4–+53.3
Sesame oil	255.2–258.8	242.5–251.9	3.3–16.3	+26.2–+48.6
Poppy oil	261.7–262.3	248.7–254.2	8.1–13.0	+35.8–+46.1
Olive oil	259.8	247.3	12.5	+34.8
Linseed oil	260.3	247.7	12.6	+35.1

* Abnormal sample: 295.4. † Abnormal sample: 255.6. ‡ Abnormal sample: 44.4.
§ Abnormal sample: 6.6.

from the Köttstorfer number, using the factor 1.366, the *soluble baryta number* (S) by subtracting I from T, and the *difference number* (D) by the formula

$$D = I - (200 + S)$$

The maximum and minimum baryta numbers of various animal and vegetable fats, as found by Avé-Lallemont,[29] Fritzsche,[30] and Bolton and Revis [31] appear in the table above.

Ethyl Ester Number

The ethyl ester number is a measure of fatty acids with 6 to 13 carbons which occur in large amount in cocoanut and palm kernel oils, in lesser amount in butter fat, but only in small amount in other common fats.

When a fat is heated with an amount of ethanolic potash insufficient to effect saponification, ethyl esters of caprillic, caproic, and lauric acids, which differ in volatility, are formed. This principle has been utilized by Hanuš [32] at the Prague Institute of Technology for the detection of cocoanut oil in butter and lard. The method as described by Hanuš and Thian [33] is as follows.

Hanuš Double Distillation Volumetric Method.[32] Process. *Alkali Treatment.* Warm at 50° for 15 minutes in an air bath 5 g. of the filtered sample contained in a 300-ml. Erlenmeyer flask, 14 cm. high, 8 cm. broad, and with a neck 2 cm. in diameter. Add immediately 30 ml. of *0.1 N ethanolic potassium hydroxide solution*, shake 4 to 6 minutes until clear, and heat for 10 minutes longer at 50° in the air bath.

Distillation of Esters. Add 2.2 ml. of *1.5 N sulfuric acid* (2 ml. = 30 ml. of *0.1 N* potassium hydroxide), 113 ml. of water, and a piece of *pumice stone*, then distil on a Rose-metal bath through a safety bulb, 15 cm. high and 3.5 cm. in diameter, and a condenser 70 cm. long. Collect separately the first (ethanolic) portion of 30 ml. (10 minutes) and the second (aqueous) portion of 100 ml. (20 to 25 minutes).

Usually cocoanut oil imparts a characteristic odor to the distillate.

Saponification and Titration. To the second portion, contained in a 500-ml. Erlenmeyer flask, add *ethanol* until the solution is clear, neutralize using *phenolphthalein indicator*, and saponify by refluxing for 45 minutes with 40 ml. of *standard 0.2 ethanolic potassium hydroxide solution.* Titrate back with *standard 0.1 N hydrochloric acid.*

Calculation. From the number of milliliters required (T), calculate the ethyl ester number (E) by the following formula:

$$E = (2 \times 40) - T$$

In doubtful cases (when the ethyl ester number is as low as 10), determine the value of the first distillate in terms of ethyl ester number, using 25 ml. of 0.2 N ethanolic alkali for the saponification.

Examples. The following figures show the application of the method.

	Second Distillate (Ethyl Ester Number)	First Distillate
Butter fat	7.1–13.4	21.8–28.1
Oleomargarine (animal fats)	1.7– 3.0	1.0– 2.7
Lard	2.7– 3.2	0.6– 1.8
Cocoanut oil (refined)	41.5–43.5	10.6–15.6
Palm kernel oil	23.1	1.5

Note. Benzoic and salicylic acids do not influence the results. Triacetin and tributyrin yield esters that pass over into the first distillate.[34]

Iodine Number

The iodine number is the percentage of iodine absorbed by the oil or fat.

Theory. Of the chemical values useful in the diagnosis of fats and oils, the iodine number is the most important. It depends

on the formation of halogen addition compounds of the glycerides of the unsaturated fatty acids and is consequently a measure of the degree of unsaturation. The results are influenced by (1) the percentage of each unsaturated fatty acid, (2) the degree of unsaturation of each acid, and (3) the mean molecular weight of the fat. Of these factors the first two exert the greater influence.

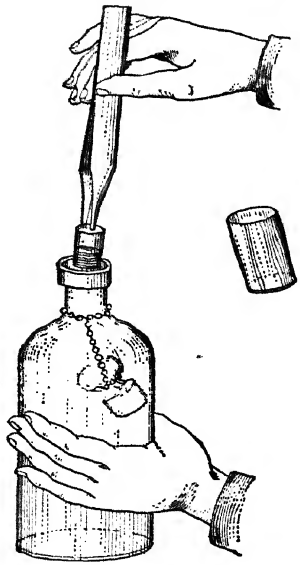


FIG. 109. Iodine Number Apparatus.

EXAMPLES. The range in values is from less than 10 (e.g., cocoanut oil) to over 200 (e.g., linseed oil). Olive oil ranges from 74 to 95, rape oil 93 to 105, cottonseed and sesame oils 103 to 115, corn (maize) oil 110 to 130, soy bean oil 115 to 145, and walnut oil 135 to 155. See also table under Values of Fats and Oils, above.

The solution employed by Hübl,[35] who devised the original method, consisting of a solution of iodine and mercuric chloride in ethanol, acted slowly and deteriorated rapidly in strength. These defects were overcome by Wijs who employed a solution of iodine chloride in glacial acetic acid and Hanuš who substituted iodine bromide for the iodine chloride, the former solution having been preferred in England, the latter in the United States, although the Committee of the American Chemical Society on Analysis of Commercial Fats and Oils [36] endorsed the Wijs solution.

Hanuš Iodine Bromide Volumetric Method.[37] APPARATUS [38] (Fig. 109). *Glass Cylinders*, 10 mm. in diameter and 15 mm. high. These may be made by cutting off flat-bottom shell vials to the proper height.

Glass-Stoppered Bottles, 400 to 500 ml. capacity.

REAGENTS. *Iodine Solution.* Dissolve 13.2 g. of pure iodine in 1 liter of glacial acetic acid and, when the solution has cooled, add 3 ml. of Br. After the addition of the Br, the halogen content, as determined by titration against thiosulfate solution, should be nearly but not quite doubled.

Standard Sodium Thiosulfate Solution, 0.1 *N*. Dissolve exactly 24.82 g. of $Na_2S_2O_3 \cdot 5H_2O$ in freshly boiled water and make up to 1 liter in a graduated flask. If the salt is pure and contains exactly 5 molecules of water, the solution will be of the proper strength and further standardizing will be merely confirmatory; 1 ml. = 0.012692 g. of iodine.

The solution may be standardized by iodine, by potassium dichromate, by potassium biniodate, or by potassium permanganate. See Introduction, Principal Standard Solutions.

Standardization by iodine, which is the oldest and believed to be the most accurate, is carried out by the writers as follows. Tare a short glass tube, such as is used for weighing out the fat (Fig. 109), together with a microscopic cover-glass; place in the tube about 0.2 g. of resublimed iodine, heat cautiously until the iodine melts, close with the cover-glass, cool in a desiccator, and weigh. Transfer the iodine together with tube and cover-

glass to a beaker and dissolve the iodine in 15 ml. of 10% KI solution. Dilute with water and add thiosulfate solution from a buret with stirring until only a yellow color remains, then add a little starch solution and continue the addition until the blue color is discharged.

To calculate the weight of iodine equivalent to 1 ml. of the thiosulfate solution, divide the weight of iodine used by the number of milliliters required for the titration, and multiply the product by 100. One milliliter of the thiosulfate solution, if exactly 0.1 N, will be equivalent to 0.0127 g. of iodine.

Standardization by dichromate [30] (the official method of the A.O.A.C.) depends on the following equation:

$$K_2Cr_2O_7 + 14HCl + 6KI = 2CrCl_3 + 8KCl + 6I + 7H_2O$$

Proceed as follows. Introduce 20 ml. of a standard 0.1 N $K_2Cr_2O_7$ solution (4.903 g. of crystallized salt dissolved in water and made up to 1 liter) into a glass-stoppered flask together with 10 ml. of 15% KI solution and 5 ml. of HCl. Add 100 ml. of freshly boiled water, cool and add slowly from a buret the $Na_2S_2O_3 \cdot 5H_2O$ solution until the yellow color has nearly disappeared. Finally add 2 ml. of 1% starch solution and continue the titration carefully until the blue color disappears. If the thiosulfate solution is exactly 0.1 N, 20 ml. will be required for the titration.

Starch Solution. Stir 1 g. of starch with 200 ml. of water, boil for 10 minutes, and cool.

Potassium Iodide Solution. Dissolve 150 g. of KI in water and dilute to 1 liter.

PROCESS. *Charge.* Weigh a flat-bottom glass cylinder, transfer to it by means of a graduated glass tube a quantity of the melted fat or oil (0.15 to 1.0 g.) sufficient to absorb not more than 40% of the iodine present in 30 ml. of the iodine solution. Do not exceed 0.15 g. of olive, cottonseed, peanut, sesame, or rape oil, 0.25 g. of lard, 0.3 g. of beef or mutton tallow, 0.4 g. of [illegible] butter, and 1.0 g. of cocoanut oil. Weigh the cylinder containing the fat or oil; if the former, after cooling to room temperature. Because of the small quantities employed, the weighing of the cylinder, both before and after adding the material, should be performed with the highest degree of accuracy which the small size of the cylinder and the non-hygroscopic character of the fatty material make possible. No desiccator need be used.

Iodine Absorption. By means of forceps carefully introduce the cylinder and contents into the glass-stoppered bottle, add 10 ml. of *chloroform* and, after complete solution is effected, introduce 30 ml. of the *iodine solution* with great care by means of a pipet. Shake gently by rotation and allow to stand in a dark place with occasional shaking for exactly 30 minutes.

Titration. Add 10 ml. of *potassium iodide solution* and 100 ml. of freshly boiled water, then titrate slowly with *standard 0.1 N thiosulfate solution*, depending for indicator first on the yellow color of the liquid and finally, when that has nearly disappeared, on the blue color obtained by adding a few drops of *starch solution*. When the titration is nearly finished, stopper the bottle after each addition of thiosulfate and shake to remove the iodine from the chloroform.

Blank. In addition to the actual analysis and along with it, blank determinations should be performed in exactly the same manner, using only the reagents.

CALCULATION. Subtract from the average number of milliliters of thiosulfate solution, obtained in closely agreeing blank determinations, the number of milliliters obtained in each actual analysis and multiply the difference by 0.0127, thus obtaining the grams of iodine absorbed. Multiply the grams of iodine absorbed by 100 and divide by the weight of material employed, thus obtaining the Hanuš iodine number, which is the per- [illegible] the oil or fat.

Wijs Iodine Monochloride Volumetric Method.[40] APPARATUS AND REAGENTS. Excepting the iodine solution, they are the same as those described under the Hanuš Method.

Iodine Solution. Weigh out 13.0 g. of resublimed iodine, dissolve in 1 liter of glacial acetic acid, and run into the solution a stream of washed and dried chlorine gas until nearly a double volume of thiosulfate solution is required to react with the same volume of the original solution. Store in paraffin-sealed amber bottles in a cool place. Do not use after 30 days.

McIlhiney[41] recommends the following procedure to remove an excess of chlorine in the iodine monochloride solution. Dissolve the iodine in the glacial acetic acid, heating if necessary. Remove a small portion and pass chlorine gas into the main portion until the halogen content of the whole solution is doubled. To remove the slight excess of chlorine in the main portion as indicated by the colorless solution, add a sufficient amount of the reserve portion until a slight iodine color appears. A slight excess of iodine is not detrimental, but an excess of chlorine must be overcome.

PROCESS. Weigh 0.1 to 0.5 g. of the sample, the smaller amount for the higher iodine numbers, into a 450-ml. glass-stoppered bottle. Dissolve in 15 to 20 ml. of *carbon tetrachloride* or *chloroform*, add 25 ml. of the *iodine solution* from a pipet. Moisten the stopper with the *potassium iodide solution* to trap any escaping chlorine or iodine, avoiding contamination of the contents. Let stand in the dark 30 minutes, along with a blank determination, and proceed with the titration and calculation as described under the Hanuš Method above.

IODINE-BROMINE NUMBER

The iodine-bromine number depends on the absorption of bromine, but the results are calculated in terms of iodine number.

Winkler Bromine Volumetric Method.[42] This procedure was found by Weiser and Donath[43] to yield results on edible fats and oils agreeing substantially with those by the Hübl method and its modifications and theoretical results on certain fatty acids (crotonic, tiglic, and cinnamic) of non-edible fats and oils to the determination of which neither the Hübl method nor its modifications are applicable. During World War I the method was recommended by Arnold[44] and others because of the cheapness of the reagents and the release of acetic acid, mercury salts, and ethanol for the manufacture of munitions.

REAGENT. *Potassium Bromate Solution,* 0.1 *N*. Dissolve 2.784 g. of K_2BrO_3 in water and make up to 1 liter.

PROCESS. *Bromine Absorption.* Introduce into a glass-stoppered bottle an amount of the sample equivalent to less than half that of the bromate solution subsequently added, (for an iodine number less than 100 use 0.2 to 0.5 g., for between 100 and 150 use 0.15 to 0.20 g., and for over 150 use 0.1 to 0.15 g.), and dissolve in 10 ml. of *carbon tetrachloride*, warming if necessary. Add 50 ml. of *0.1 N potassium bromate solution*, then 1 g. of *solid potassium bromide.* Shake gently until the latter has dissolved, add 10 ml. of *10% hydrochloric acid*, stopper at once, shake again gently, and allow to stand in the dark for 0.5 to 2 hours with occasional shaking. Fish oils require 4 hours.

Titration. Remove the stopper, add at once 10 to 15 ml. of *10% potassium iodide solution*, shake, and titrate with *0.1 N sodium thiosulfate solution.* It is unnecessary to use starch paste except when working with artificial light or when the solution of the fat has a decided color.

Blanks. Conduct blank determinations and make the necessary deduction.

CALCULATIONS. See Hübl Method for details of calculation of the percentage of halogen absorbed in terms of iodine.

I. Köpke Arsenite Modification.[45] By the substitution of standard 0.1 *N* sodium arsenite for potassium iodide, also of standard 0.1 *N* potassium bromate for thiosulfate solution, Köpke dispenses with all iodometric reagents.

REAGENT. *Sodium Arsenite Solution*, 0.5 *N*. Dissolve 5 g. of As_2O_3 and 2.5 g. of NaOH in 50 ml. of water, filter through a wad of cotton, wash, and make up to 200 ml.

PROCESS. *Bromine Absorption.* Use grams of oil or fat for iodine numbers as follows: 0.15 to 0.20 for 200 to 150; 0.20 to 0.30 for 150 to 100; 0.3 to 0.6 for 100 to 50; 0.6 to 1.0 for 50 to 20; and 1 to 2 for 20 or less. Dissolve in 10 ml. of *carbon tetrachloride* and proceed as in the original method with 50 ml. of *0.1 N potassium bromate*, 1 g. of coarsely *powdered potassium bromide*, and 10 ml. of *10% hydrochloric acid*. Wet the ground surface of the stopper with concentrated phosphoric acid to avoid loss.

Bromate Titration. After the absorption is complete, lift the stopper slightly and add 10 ml. of *0.5 N sodium arsenite solution*, shake until decolorized, then add 20 ml. of *hydrochloric acid* and titrate in daylight with *0.1 N potassium bromate solution* to a pale yellow color or, if artificial light is necessary, employ 2 drops of *0.2% indigo-carmine indicator*, shaking well after each addition.

Blank. Make a blank determination on the reagents, using the same amounts as in the actual analysis, excepting the 0.1 *N* potassium bromate solution, of which use only 25 ml., diluted with 25 ml. of water.

CALCULATION. Calculate the iodine-bromine number (*N*) by the following formula:

$$N = \frac{[(50 + T) - (25 + T')] \times 1.27}{C}$$

in which *T* and *T′* are the number of milliliters of 0.1 *N* potassium bromate solution used in the titration of the unknown and the blank respectively, and *C* is the weight of the charge in grams.

II. Sabalitschka and Dietrich Simple Modification.[46] APPARATUS. The only apparatus required for the process is a *glass plate* 5 x 14.5 cm., *another plate* of smaller size, and a glass-stoppered *cylindrical bottle*.

PROCESS. Place about 0.2 g. of the oil or melted fat on the larger plate near one of the shorter sides, then by means of the other plate distribute the charge uniformly over the surface, leaving, however, on all sides a narrow margin (breadth 1 to 2 cm.) for handling. Weigh, place in the bottle together with a boat containing 8 to 10 drops of *bromine*, and allow to stand for 1 hour. Then warm the plate for 2 to 3 hours at 60° to remove the excess of bromine, cool, and weigh.

CALCULATION. Determine the iodine-bromine number (*N*) from the formula

$$N = \frac{100 \times A \times I}{C \times B}$$

in which *A* is the weight of bromine absorbed, *I* is the atomic weight of iodine, *B* is the atomic weight of bromine, and *C* is the weight of the charge.

The results are slightly, but uniformly, higher than those by the Hübl method.

HEXABROMIDE NUMBER

The hexabromide number is a measure of the content of the highly unsaturated fatty acids in linseed and peanut oils.

Determination of iodine and bromine numbers is not the only practical application of halogen absorption by unsaturated fats and oils. Hehner and Mitchell[47] based a method on the insolubility of bromine addition products of linoleic acid of linseed oil and of arachidonic acid of peanut oil which was improved by Eibner.[48]

This method is not to be confused with the hexabromide test for fish oils (see Fish Oils below).

Tolman Centrifugal Method.[49] Tolman devised a simple method embodying the new feature of centrifuging instead of filtering.

PROCESS. *Bromination*. Dissolve a weighed portion of the sample or its fatty acids in 25 ml. of *absolute ether*, contained in a weighed centrifuge tube, add a drop of *acetic acid*, cool in an ice bath to 2 to 3°, and then add an excess of *bromine* dropwise with continual stirring to avoid overheating. Cool for 30 minutes in the ice bath, centrifuge at 1200 r.p.m. for 2 to 3 minutes, cool again, and decant the clear ether solution.

Ether Extraction. Thoroughly mix with 10 ml. of cold *absolute ether*, cool, whirl, and decant, repeating the treatment. Evaporate the ether, dry in a boiling water bath for 30 minutes, cool, and weigh the hexabromides thus prepared which are white with a slight amber tint.

EXAMPLES. On fish and high carbon liver oils treated directly, values from 19.57 to 57.54 and on fatty acids 26.08 to 36.12 were obtained, but on linseed oil only 28.82 and 36.04 respectively. Doubtless the method would yield higher results if specially purified ether saturated with hexabromine were used as in the Steele and Washburn method.

Steele and Washburn Gravimetric Method for Linseed Oil.[50] The main features of this method, developed at the U. S. Bureau of Standards, are (1) addition of bromine to the fatty acids of the sample in a solvent in which the resulting hexabromide formed is soluble, (2) addition of amylene to remove excess bromine, (3) evaporation of the solvent, and (4) isolation of hexabromide free from foreign bromides by absolute ether saturated with hexabromide.

REAGENTS. *Chloroform*. Remove ethanol from commercial chloroform by shaking with several portions of water and dry by allowing to stand overnight with granular $CaCl_2$. Decant, distil, add 3 ml. of absolute ethanol to every 100 ml. of the distilled chloroform, and store in glass-stoppered brown bottles.

Bromine Solution. Mix fresh each day 1 part by volume of bromine free from solids with 2 parts of the chloroform.

Wash Ether. Purify ordinary ethyl ether by shaking with 4 successive portions of one-tenth its volume of ice cold distilled water. Dry overnight with $CaCl_2$, decant, filter, and warm under a reflux condenser with thin slices of metallic sodium until evolution of gas ceases and fresh slices of sodium remain untarnished. Distil, using a dry bottle as receiver. Add an excess (3 g. or more) of finely powdered hexabromide from a previous determination or prepared as follows.

Dissolve 5 g. of the fatty acids of linseed oil in 15 to 20 ml. of chloroform in a centrifuge tube, cool in a freezing mixture, and add slowly with shaking bromine solution until a slight red color persists, then a few drops of amylene to combine with the excess of bromine. Whirl, pour off the clear solution, rub up the precipitate with 20 ml. of cold absolute ether, whirl, pour off the ether, repeat the washing 3 times, and dry.

After shaking the ether with the hexabromide of the fatty acids at intervals during 2 to 3 hours, or allowing to stand overnight, cool in ice water to 0 to 2°, keep at that temperature for 3 hours, and filter rapidly through a pleated paper into a dry bottle. Keep tightly stoppered.

Amylene. Prepare from amyl alcohol as described by Adams.[51] The University of Illinois and the Eastman Kodak Company supply the chemical of suitable quality.

PROCESS. *Fatty Acids Separation*. To 50 g. of the sample in a 1.5-liter flask, add 40 ml. of *sodium hydroxide solution* (specific gravity 1.4) and 40 ml. of *ethanol*, heat on a steam bath for 30 minutes, and dilute with 1 liter of hot water. Remove the ethanol over a steam bath (2 to 3 hours) or a free flame (0.5 hour) while passing a current of *carbon dioxide* into the flask, the entrance tube being slightly above the liquid; increase the current if the foaming is excessive. If heated over a free flame, a capillary "boiler" will mitigate bumping.

Cool and add *1 + 1 hydrochloric acid* to acid reaction. Insert a 3-hole stopper carrying 3 tubes; the one reaching just above the liquid is the inlet for carbon dioxide; the second, just through the stopper, is the outlet for the gas; and the third, extending to the bottom, is the outlet for removal, by pressure of the gas, of the aqueous liquid by closing the second tube. Boil in the current of *carbon dioxide* until the fatty acids become clear and remove the aqueous liquid as completely as possible in the manner indicated without loss of fatty acids. Raise the stopper, add 500 ml. of hot water, shake well, allow to settle, and siphon out the aqueous liquid as before, repeating the operation until all mineral acid is removed. Before the last washing boil until the fatty acids are clear and after the washing remove the last of the aqueous liquid with a pipet. Filter the hot fatty acids under an evacuated bell-jar. Store in a well-stoppered bottle.

Hexabromides Preparation. Dissolve 1 g. of the fatty acids contained in a centrifuge tube, 16 cm. long and 2.5 cm. in diameter (tared with a stirring rod), in 10 ml. of washed and dried *chloroform*. Cool to −5° in a freezing bath of a little *dilute hydrochloric acid* and finely cracked ice. Add *bromine solution* from a buret, 1 or 2 drops per second with shaking, avoiding direct sunlight, until an orange color persists (this requires about 1 ml.), then add rapidly 0.5 ml. additional. Shake well, cool further in the freezing bath for 10 minutes, remove from the bath, and add *amylene* dropwise with shaking until the bromine color disappears; this usually requires 5 or 6 drops, although a slight excess is not detrimental.

Connect the tube with a vacuum pump, exhaust at 40-mm. mercury pressure, and evaporate the chloroform, warming in water at 50 to 60°, constantly shaking to prevent bumping, and at the end rotating to spread the viscous mass. Finally heat at 55 to 60° for 15 minutes with continued suction.

Disconnect the pressure, cool in an ice-water bath, and add the contents of one of four test tubes previously filled to a 20-ml. mark with *wash ether* and cooled to 0° in the ice-water bath. Rub up the lumps thoroughly with the rod, return to the ice bath for 2 minutes, whirl until the supernatant liquid above the hard deposit is clear, cool again for 2 minutes, and pour off the wash ether. Repeat the washing, centrifuging, and decanting with the 3 remaining 20-ml. portions of *wash ether*.

After the final washing, distribute the hexabromide on the sides of the tube, warm at 50 to 60° until most of the ether has evaporated, and heat further at 60 to 70° for 15 minutes with suction. Remove from the bath, wipe with a cloth, weigh at once, then dry to constant weight at 100 to 110°.

CALCULATION. Multiply the weight of the hexabromide (which should be pure white) by 100 and divide by the weight of the fatty acids to obtain the percentage of hexabromide; this is the hexabromide number.

THIOCYANOGEN NUMBER

The thiocyanogen number in conjunction with the iodine number permits the calculation of linoleic, oleic, and saturated fatty acids by formulas. See below.

Kaufmann Volumetric Method.[52] Martin and Stillman Modification.[53] This modification, used in the Procter and Gamble laboratory, is endorsed by the Committee on Analysis of Commercial Fats and Oils of the American Chemical Society.[54]

REAGENTS. *Lead Thiocyanate.* To a solution of 250 g. of KSCN in 500 ml. of water, add slowly with stirring a solution of 250 g. of $Pb(CH_3COO)_2 \cdot 3H_2O$ in 500 ml. of water. Collect the crystalline precipitated lead thiocyanate on a Büchner funnel and wash successively with water, ethanol, and ether. Dry first by suction on the funnel, then for 8 to

10 days over P_2O_5. The lead thiocyanate should be discarded if the color is other than greenish or yellowish white or if kept over 2 months.

Anhydrous Acetic Acid. Place 2 liters of glacial acetic acid and 100 ml. of acetic anhydride (90 to 100%) in a 3-liter flask, with a large test tube set in the neck through which cold water passes to act as a condenser, and reflux for 3 hours heating at 135° in an oil bath. Cool and store in dry bottles.

Thiocyanogen Solution, 0.2 N. To a suspension of 50 g. of dry $Pb(SCN)_2$ in 500 ml. of anhydrous acetic acid contained in a previously cleaned and dried glass-stoppered bottle, add slowly with shaking until decolorized a solution of 5.1 ml. of bromine in 500 ml. of anhydrous acetic acid. After the precipitated $PbBr_2$ and excess of $Pb(SCN)_2$ have settled, filter as rapidly as possible on a Büchner funnel with a layer of filter paper pulp previously dried at 105°. Refilter through the same filter paper layer. Store in glass-stoppered brown bottles at 15 to 21°. Reject when titration of 25 ml. with thiosulfate shows a decrease in a 24-hour interval of 0.2 ml.

PROCESS. *Thiocyanogen Treatment.* Accurately weigh into a well-dried glass-stoppered flask or bottle 0.1 to 0.3 g. of the sample, regulating the quantity according to the material so that the excess of thiocyanogen is at least as much as that absorbed and preferably not more than one and one-half times that amount. Add from a pipet 25 ml. of the *thiocyanogen solution* and dissolve by a gentle swirling motion.

Titration. After standing for 24 hours in the dark at 18 to 21°, add 1 g. of dry *powdered potassium iodide*, swirl rapidly for 2 minutes, dilute with 30 ml. of water, and titrate the liberated iodine with *0.1 N sodium thiosulfate solution*, using *starch solution* as indicator (see Iodine Number above).

Blank. Conduct 3 blank determinations alongside of the actual analysis.

CALCULATION. Obtain the thiocyanogen number (*TV*) by the following formula:

$$TV = \frac{0.01269(B - U) \times 100}{G}$$

in which B and U are the number of milliliters of 0.1 N thiosulfate solution required for the average of the blanks and the unknown respectively, and G is the grams of the charge.

NOTE. Wiley and Gill [55] have demonstrated that by using 0.4 g. of the sample instead of 0.2 g. and 50 ml. of *thiocyanogen solution* the accuracy of the method is greatly increased.

LINOLENIC, LINOLEIC, OLEIC, AND RATED GLYCERIDES AND ACIDS

Calculation Formulas.[56] The following revised formulas, replacing those previously adopted, are recommended by the Committee on Standard Methods of Analysis of the American Chemical Society. The methods are as given above, but the excess of reagent for the thiocyanogen number is increased from 100 to 150% to 150 to 200%. When solid samples will not conveniently dissolve in the standard reagent, 60 + 40 ascetic acid-carbon tetrachloride may be used.

A. Iodine number (IV) and thiocyanogen number (TV) determined on the fatty acids respectively as follows: linolenic acid (X) 273.7 and 167.1, linoleic acid (Y) 181.1 and 96.7, oleic acid (Z) 89.9 and 89.3, and saturated acids (S) 0 and 0.

(a) Linolenic acid absent:

$$181.1Y + 89.9Z = 100\ \mathrm{IV}$$

$$96.7Y + 89.3Z = 100\ \mathrm{TV}$$

$$S = 100(Y + Z)$$

$$Y = 1.194\ \mathrm{IV} - 1.202\ \mathrm{TV}$$

$$Z = 2.421\ \mathrm{TV} - 1.293\ \mathrm{IV}$$

$$S = 100 - (Y + Z)$$

(b) Linolenic acid present:

$$273.7X + 181.1Y + 89.9Z = 100\ \mathrm{IV}$$
$$167.1X + 96.7Y + 89.3Z = 100\ \mathrm{TV}$$
$$X + Y + Z = 100 - S$$

$$X = 1.5902\ \mathrm{TV} - 0.1290\ \mathrm{IV} + 1.3040S - 130.40$$
$$Y = 1.3565\ \mathrm{IV} - 3.2048\ \mathrm{TV} - 1.6423S + 164.23$$
$$Z = 1.6146\ \mathrm{TV} - 1.2275\ \mathrm{IV} - 0.6617S + 66.17$$

B. **Iodine and thiocyanogen numbers determined on mixed triglycerides and calculation made for hypothetically pure triglycerides as follows:** linolenin (X) 261.8 and 159.8, linolein (Y) 173.3 and 92.5, olein (Z) 86.0 and 85.5, and saturated and unsaponifiable glycerides (S) 0 and 0.

(a) Linolenin absent:

$$Y = 1.246\ \mathrm{IV} - 1.253\ \mathrm{TV}$$
$$Z = 2.525\ \mathrm{TV} - 1.348\ \mathrm{IV}$$
$$S = 100 - (Y + Z)$$

(b) Linolenin present:

$$261.8X + 173.3Y + 86.0Z = 100\ \mathrm{IV}$$
$$159.8X + 92.5Y + 85.5Z = 100\ \mathrm{TV}$$
$$X + Y + Z = 100 - S$$

$$X = 1.6610\ \mathrm{TV} - 0.1332\ \mathrm{IV} + 1.3056S - 130.56$$
$$Y = 1.4137\ \mathrm{IV} - 3.3449\ \mathrm{TV} - 1.6441S + 164.41$$
$$Z = 1.6839\ \mathrm{TV} - 1.2805\ \mathrm{IV} - 0.6615S + 66.15$$

Acetyl Number

The acetyl number is theoretically a measure of hydroxylation of the glycerides, but actually other elements enter into the reactions. Hydroxy fatty acids are fatty acids in which one or more of the hydrogen atoms of the chain are replaced by the hydroxyl group: the hydrogen of these hydroxyl groups in turn may be replaced by acetic anhydride. On this latter reaction is based the determination of acetyl number which is the *milligrams of potassium hydroxide required for the neutralization of the acetic acid obtained on saponifying 1 g. of a fat or oil thus acetylated.*

The following formulas show the relation of monohydroxyoleic acid (ricinoleic acid) to oleic acid, and of dihydroxystearic acid to stearic acid:

$C_nH_{2n-2}O_2$:

$CH_3(CH_2)_7$–CH CH–$(CH_2)_7$–COOH,
Oleic acid.

$C_nH_{2n-2}O_3$:

$CH_3(CH_2)_7$–CH=COH–$(CH_2)_7$–COOH,
Monohydroxyoleic acid.

$C_nH_{2n}O_2$:

$CH_3(CH_2)_7$–CH_2–CH_2–$(CH_2)_7$–COOH,
Stearic acid.

$C_nH_{2n}O_4$:

$CH_3(CH_2)_7$–CHOH–CHOH–$(CH_2)_7$–COOH,
Dihydroxystearic acid.

Acetyl Numbers of Edible Oils and Fats. This value is not so often determined as the other physical and chemical values treated in this volume. Even if the numerous results by the Benedikt-Lewkowitsch method are included, there are many gaps in the literature and there is a wide range in the results due to causes not characteristic of the individual oils that led Lewkowitsch to class this value as a variable, not a constant.

In general, the acetyl number of edible fats and oils seldom exceeds 20 and never approaches that of castor oil of the inedible class which reaches 150. The values for cottonseed and sesame oils range usually from 10 to 15 and those for cacao butter, cocoanut and palm nut oils, and for common animal fats range from 1 to 8. The figures obtained by the André-Cook method are not strictly comparable with those by the earlier meth-

ods, even when the latter are corrected as suggested by Lewkowitsch.

Benedikt-Lewkowitsch Methods.[57] The determination of the acetyl number was first proposed by Benedikt [58] and was long carried out as described by Lewkowitsch,[59] either by the distillation method, in which the acetic acid liberated from the acetylated oil or fat by *sulfuric acid* is distilled and titrated, or by the filtration method, in which the liberated fatty acids are filtered, washed, and weighed. Both methods are not only laborious, but also inaccurate, since, as pointed out by Lewkowitsch, in addition to hydroxy acids, free alcohols, such as cholesterol and phytosterol, mono- and diglycerides, and unknown products of rancidity combine with acetic anhydride. Only when triglycerides alone are present is the result a characteristic or true value, as distinguished from a variable.

André-Cook Saponification Volumetric Method. At least some of the inaccuracies of the Benedikt-Lewkowitsch method are overcome by the calculation of the acetyl value from the saponification values of the sample before and after acetylation, employing the formula of André [60] or, more conveniently, its simplified form as derived by Cook [61] and given below.

Process. *Acetylation.* As adopted by the A.O.A.C. and the American Chemical Society Committee on Analysis of Commercial Fats and Oils, the acetylation is carried out essentially as described by Lewkowitsch,[62] except that an equal volume of acetic anhydride is used.

Reflux 50 g. of the sample with 50 g. of freshly distilled *acetic anhydride* for 2 hours, pour the mixture into 500 ml. of boiling water, and boil for 15 minutes while passing a slow stream of air or *carbon dioxide gas* into the liquid to prevent bumping. When the two liquids separate, siphon off the aqueous portion and repeat the addition of water, boiling, and siphoning the oily layer twice; then *litmus paper* should show that all the acetic acid has been removed. Lewkowitsch warns against overwashing, which causes dissociation.

Allow the solution to cool, remove the aqueous layer, and transfer the acetylated oil to a separatory funnel. Shake twice with 200 ml. of warm water, remove the aqueous liquid as completely as possible, add 5 g. of *anhydrous sodium sulfate* to the acetylated oil, and shake at intervals for 1 hour to remove the water. Filter through a dry pleated paper, preferably in an air oven at 100 to 110°, and continue the heating until all water is removed.

Saponification. Weigh into separate 250-ml. Erlenmeyer flasks 2 to 2.5 g. of the sample before and after acetylation and reflux for 1 hour with exactly 25 ml. of *ethanolic potassium hydroxide solution* as in the determination of saponification number.

Titration. Titrate the warm solutions with *standard 0.5 N hydrochloric acid*, using *phenolphthalein indicator*. Also titrate two or more accurately measured portions of 25 ml. each of the ethanolic potassium hydroxide solution.

Calculation. Deduct from the average of the readings on the alkali solution alone the readings on the acetylated and unacetylated oils and calculate the saponification number of each, then calculate the acetyl number by the Cook formula as follows:

$$A = \frac{S' - S}{1 - 0.001S \times (42/56)} = \frac{S' - S}{1 - 0.00075S}$$

in which A is the acetyl number, S is the saponification number of the sample, S' is the saponification number of the acetylated sample, 56 is the molecular weight of potassium hydroxide, and 42 is the increase in its molecular weight on acetylation.

Hydroxyl Number

The hydroxyl number, like the acetyl number, is a measure of hydroxylization, but it is a

function of the fat, not of the acetylated fat, and is independent of the reagents and process. In deference to long usage, however, the value is in terms of milligrams of potassium hydroxide corresponding to the acetyl of the acetylated fat, hence the two numbers may be calculated one from the other.

Elsbach Acetic Anhydride Gravimetric Method.[63] The method in principle and technique is simplicity itself, but does not appear to have attracted general attention.

PROCESS. Weigh 2 to 5 g. of the sample into a round-bottom flask and heat in an air bath at 100°, in a stream of *carbon dioxide* dried by passing through *sulfuric acid*, to constant weight; this requires about 1 hour. Displace the gas by air before weighing. Acetylate with 4 to 6 ml. of *acetic anhydride* by heating for 30 minutes under a reflux condenser. After acetylizing, heat again in a stream of *carbon dioxide gas* for 1.5 to 3 hours until the excess of acetic anhydride is removed and weigh.

CALCULATION. The hydroxyl number (H) and the acetyl number (A) are obtained by the following formulas:

$$H = G \times \frac{56{,}110}{42{,}016 \times 100} = 13.354G$$

$$A = \frac{13.354G}{1 + 0.01G}$$

in which G is the percentage gain in weight of the sample over the dry weight by acetylizing.

EXAMPLES. Elsbach reports figures on hydroxyl number and acetyl number respectively as follows: castor oil 166.6 and 148.3; sesame oil 5.7 and 5.6; cocoanut oil 5.1 and 5.1; and boiled linseed oil 20.5 and 20.2.

Roberts and Schuette Sealed-Tube Volumetric Modification.[64] Except when filtration to remove soluble acids is necessary, the entire process is carried out in the presence of the acetylated product. The originators (both of the University of Wisconsin) further state that the use of the sealed tube is believed to entail no unusual hazard to the operator.

APPARATUS. *Reaction Tubes.* Draw out in the blast lamp flame the middle portion of 300-mm. lengths of soft glass tubing 10 mm. in diameter, separate into two equal lengths, and seal the larger ends.

REAGENT. *Acetic Anhydride Solution*, 90%, freshly distilled. Standardize monthly and express the standard in terms of milligrams of KOH per milliliter of reagent.

PROCESS. *Acetylation.* Adjust to (1) the probable hydroxyl number, (2) the grams of the charge, and (3) the grams of acetic anhydride respectively as follows: 0 to 50, 5.0 g. and 1.5 g.; 50 to 100, 2.5 g. and 1.5 g.; 100 to 200, 2.5 g. and 2 g.

Tare the tube, add the *90% acetic anhydride solution* from a calibrated capillary pipet, weigh, add the charge, and reweigh. Add solid fats in pellet form. Seal the tube, heat in an oven at 120° for 10 minutes, shake, then return to the oven, and allow to rest in a horizontal position for 1 hour. Cool the tube, open, and pour the contents into 50 ml. of water contained in a 500-ml. Erlenmeyer flask. Rinse several times, first with cold, then with hot water, and dilute to about 200 ml. Swirl the flask, add exactly 50 ml. of *0.5 N potassium hydroxide solution* and a few *glass beads*, attach a reflux condenser, and bring nearly to boiling over a free flame. Rinse down the condenser tubes with 50 ml. of water, wash off the tip, swirl, and cool.

Titration. Complete the addition of *standard alkali*, using 20 drops of *thymol blue solution* (or 10 drops of *phenolphthalein solution*) as indicator.

Blank. If the sample is normal, a blank need not be made. If, however, it contains free soluble acids or it is rancid, it is necessary to filter and thoroughly wash with hot water prior to titration. In making the blank determination, use a weighed portion of the unacetylated oil, wash thoroughly with hot

water on a moistened paper, and titrate the washings.

CALCULATION. Convert all buret readings into equivalent milligrams of potassium hydroxide and calculate the hydroxyl number (H), also the acetyl number (A), as follows:

$$H = B - C$$

$$A = \frac{H}{1 + 0.00075H}$$

in which B and C are the potassium hydroxide values in milligrams respectively of the acetic anhydride used for acetylation and the excess obtained by titration, and G is the weight of the sample in grams.

Kaufmann and Funke Acetyl Chloride Volumetric Method.[65] The substitution of acetyl chloride for acetic anhydride in the following procedure, developed at Münster University, has the advantage over the foregoing of a more rapid reaction and eliminates the error due to the formation of anhydrides.

PROCESS. *Acetylation.* Place in a flask a weight of the sample that will leave at least 100% of the acetyl chloride solution in excess. Dissolve in 5 ml. of dry *pyridine* and add 5 ml. of *1 to 1.5 M acetyl chloride solution in toluol* from a buret with a drawn-out delivery tube introduced beneath the solution. Take care that none of the solution remains attached to the end of the tube. Close the flask with a rubber stopper and heat in a water bath for 5 minutes at 65 to 70° with shaking. Cool under a tap, add 10 ml. of water, shake vigorously, and reflux for 5 minutes.

Titration. Cool, rinse the condenser with water, add *phenolphthalein indicator*, and titrate with *standard 0.5 N potassium hydroxide in ethanol.*

Blank. Make a blank determination on the reagents.

Acid Number. Determine the acidity of the sample by the usual process and express the result as the acid number, that is, the milligrams of potassium hydroxide required to neutralize the free fatty acids of 1 g. of the sample.

CALCULATION. Obtain the hydroxyl number (H) by the following formula:

$$H = \frac{(B - S) \times 28.055}{G}$$

in which B and S are the milliliters of standard alkali used respectively in the blank and actual determination, G is the weight in grams of the charge, and A is the acid number, separately determined.

CRITICAL TEMPERATURE OF DISSOLUTION

Valenta Acetic Acid Method.[66] Among the methods for fats which may be classed with the Maumené method as depending on strict adherence to conventional details for success are the original critical temperature of dissolution (turbidity) method of Valenta, who used acetic acid, the later method of Crismer,[67] who substituted ethanol for acetic acid, and the modification of the Crismer method by Fryer and Weston,[68] who used a 1 + 1 mixture of 92% ethanol and 100% amyl alcohol.

The results by different analysts on a variety of oils and fats are not such as to inspire confidence, although each agreed with himself; the methods are, however, of value when applied to a sample in question and a sample of unquestionable purity at the same time. The Valenta method has proved to be dependable when used in this manner for the detection of foreign fats in chocolate and cocoa products. See Part II, I3.

DIENE NUMBER; MALEIC ANHYDRIDE NUMBER

The diene number of Kaufmann and Baltes[69] *and the maleic anhydride number of Ellis and Jones*[70] *were regarded as measures of the*

reaction of the double bonds of the fat molecule until Bruce and Denley[71] *showed that the reaction also involves hydroxyl groups already in the oil or formed during oxidation; furthermore that the diene number and the maleic anhydride number are not necessarily identical.*

Bickford, Dollear, and Markley[72] confirmed Bruce and Denley's findings and showed that certain hydroxylated compounds and oils exhibiting characteristics of such compounds, when acetylated, give diene and maleic anhydride numbers of practically zero. On the other hand, linseed and soy bean oils, with initially low values, rise appreciably after acetylation. These and other data indicate a shift of the polyethenoid bonds toward a conjugated system.

The two numbers, like most other fat values, will doubtless be of greatest significance when considered in conjunction with other numbers.

Kaufmann and Baltes Maleic-Acetone Volumetric Method.[69] PROCESS. *Maleic Anhydride Treatment.* Weigh 0.1 to 0.15 g. of the oil, in reverse order to the degree of reaction, into a small glass sample tube and introduce the tube into a 20 ml. Jena glass bomb. Add to the oil 10 ml. of *1% resublimed maleic anhydride solution in acetone,* seal, and heat 20 hours at 100° in a paraffin bath. Cool, open the bomb, and rinse the contents with 80 ml. of *carbon dioxide-free water* into a 250-ml. Erlenmeyer flask, adding a little *sodium chloride* to promote separation of the fat for filtration.

Titration. Allow to stand 6 to 8 hours, filter, and titrate the filtrate with *standard 0.1 N sodium hydroxide solution*, using *1% ethanolic phenolphthalein* as indicator.

Blank. Run a blank along with the actual analysis and obtain by difference the number of milliliters of standard alkali representing the maleic anhydride taking part in the reaction.

CALCULATION. Assuming that one molecule of maleic anhydride reacts with one double bond, the diene number (D) is calculated in accordance with the following equation:

$$D = \frac{1.269 \times A}{G}$$

in which A is the number of milliliters of 0.1 N sodium hydroxide solution as found by subtracting the milliliters used in the actual analysis from the number used in the blank analysis and G is the weight in grams of the charge.

Ellis and Jones Maleic-Toluene Volumetric Methods.[73] The methods, as used for tung oil, follow.

Method I. REAGENTS. *Maleic Anhydride Solution*, approximately 6% in toluene. Dissolve 60 g. of pure commercial maleic anhydride in warm toluene, cool, and dilute with toluene to 1 liter. Prepare at least a day before needed and filter on a fast filter paper immediately before using.

Sodium Hydroxide Solution, 1.0 N. Standardize against 1 g. of pure maleic acid dissolved in 100 ml. of water, using phenolphthalein as indicator.

PROCESS. *Maleic Anhydride Treatment.* Weigh accurately about 3 g. of the oil into a dry 250-ml. volumetric flask with ground-glass neck. Pipet into the flask 25 ml. of freshly filtered *maleic anhydride solution*, add a pinch of *powdered pumice*, and connect to the reflux condenser, dusting the ground-glass connection with very fine *powdered graphite.* Boil very gently for 3 hours, cool a few minutes, add a pinch of *pumice* through the top of the condenser, and wash down into the flask with 5 ml. of water, then continue the boiling for 15 minutes more.

Fat Extraction. Cool to room temperature, pour 5 ml. of *ether* through the condenser into the flask, and then 20 ml. of water. Detach the flask, remove to a separatory funnel, using 20 ml. of *ether* in 3 portions, then 25 ml. of water, also in 3 portions. Shake, allow to separate, and run off the lower liquid into a 250-ml. flask. Extract the residual liquid in

the funnel successively with 25 ml. and 10 ml. of water.

Titration. Titrate the mixed aqueous liquids with *1.0 N sodium hydroxide solution*, using *phenolphthalein indicator*.

Blank. Conduct a blank determination and obtain the volume representing the maleic anhydride by difference.

CALCULATION. Calculate the maleic anhydride number (M) by the formula:

$$M = \frac{12.692 \times A}{G}$$

in which A is the number of milliliters of 1.0 N sodium hydroxide solution obtained by difference and G is the weight in grams of the charge.

Method II. Proceed as in Method I, except that 0.2 ml. of approximately *0.1 N iodine solution in toluene* is added as a catalyst to the contents of the flask before attaching to the condenser. Heat as before, but only for 1 hour instead of 3.

CALCULATION. As in Method I above.

c. Chemical Constituents

MOISTURE AND VOLATILE MATTER

The four methods which follow are those adopted by the American Chemical Society Committee on the Analysis of Commercial Fats and Oils.[74]

Vacuum Oven Method. APPARATUS. *Powell or F.A.C. Vacuum Oven* (Fig. 110).

Standard Moisture Dish. Glass-lipped beaker about 6 to 7 cm. in diameter and 4 cm. deep.

PROCESS. Weigh out 5 g. (±0.2 g.) of the prepared sample into a standard glass moisture dish. Dry to constant weight in the vacuum oven at not less than 20° or more than 25° above the boiling point of water at the working pressure of 100 mm. or less as shown in the following table:

Mm.	To 1°	+20°	+25
100	52	72	77
90	50	70	75
80	47	67	72
70	45	65	70
60	42	62	67
50	38	58	63
40	34	54	59

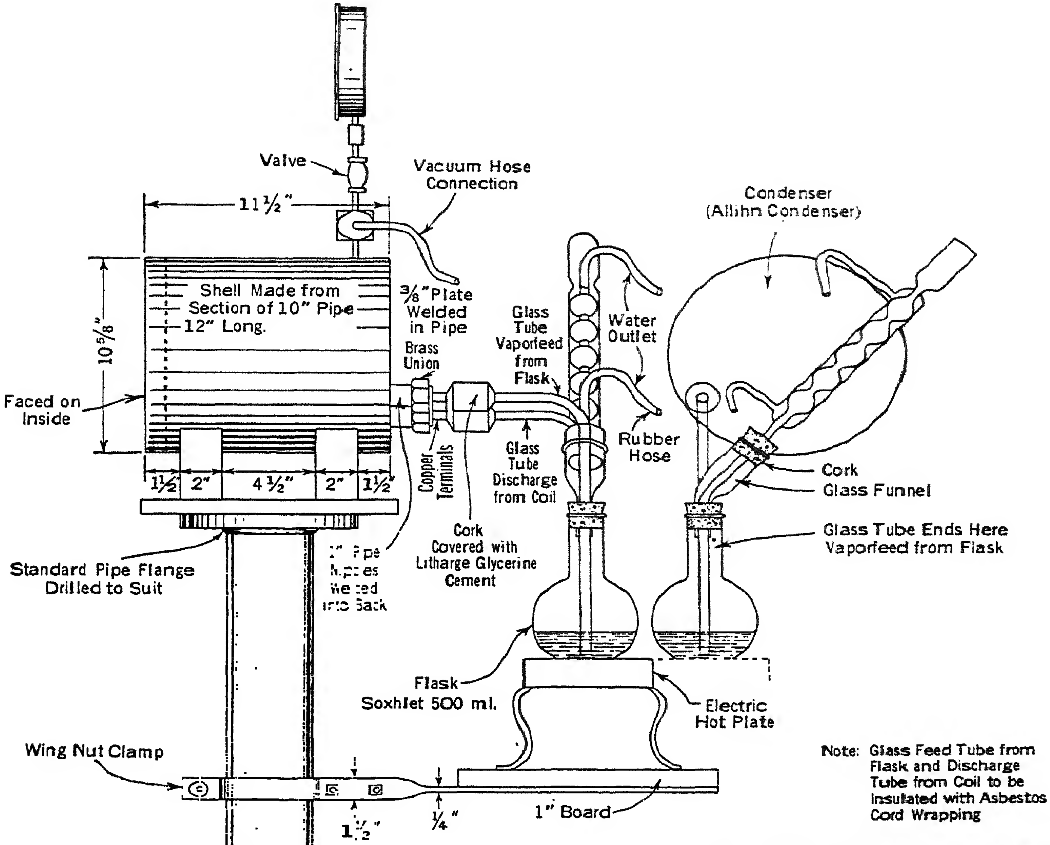
The Powell oven is heated by a brass coil in which circulates carbon tetrachloride which boils:[75] 76.1° at 743 mm., 71.0° at 663 mm., 65.0° at 559 mm., 62.0° at 506 mm., 58.5° at 443 mm., 54.8° at 386 mm., 50.0° at 328 mm., 46.3° at 282 mm., 42.0° at 236 mm., 31.0° at 149 mm., and 25.0° at 116 mm.

Air Oven Method. This method is not considered to be even approximately accurate for drying or semi-drying oils (cottonseed, maize, soy bean, linseed oils, etc.) and those of the cocoanut oil group containing free acid (cocoanut and palm kernel oils, etc.). Most fats and oils, however, yield on drying in a well-constructed and well-ventilated air oven at 105 to 110° results comparable with the standard vacuum oven method.

Hot Plate Method. This method, although not suited for certain abnormal greases, is recommended by the Committee for routine control work on ordinary fats and oils, including butter, oleomargarine, and high-acid cocoanut oil.

PROCESS. Heat with rotation 5 to 20 g. of the prepared sample in a tared glass beaker or casserole on a heavy asbestos board over a burner or hot plate, avoiding a temperature over 130°. Toward the end of the process, steam bubbles cease to rise through the liquid fat and later there is an absence of foam. Finally heat cautiously for a moment at incipient smoking. Cool in a desiccator and weigh.

Kingman Distillation Volumetric Method. This method, like the Gray Method described in Part II, G7 (Fig. 172), depends on



Courtesy of Ind. Eng. Chem. 1926,, 18 1347

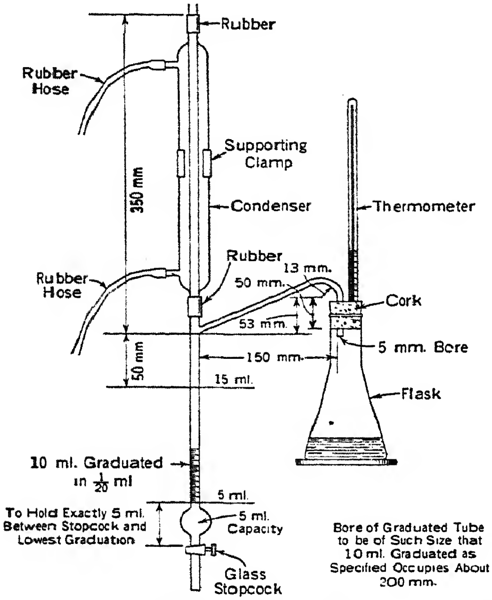
Fig. 110. F.A.C. Standard Vacuum Oven.

measuring the volume of the distillate (moisture only) from the fat. It may be carried out without a chemical balance, since the charge may be weighed out on an inexpensive open-pan balance accurate to 0.1 g.

Apparatus. The parts together with dimensions are clearly shown in Fig. 111.

Process. Weigh into a 500-ml. Erlenmeyer flask 50 to 200 g. of the sample, the larger amounts for the lower percentages; thus, for 1% or less use 200 g., for 1 to 5% use 100 g., and for over 5% smaller amounts. Add about 100 ml. of *acetylene tetrachloride* and distil until 25 to 40% of the solvent has passed over; draw off the solvent from time to time. During the rapid rise in temperature to 144 or 145°, following the gradual rise from 95 to 100° from the initial boiling temperature of 80°, slow down the heating to avoid boiling away the water already distilled. Cool and wash down the condenser and connecting tube into the graduated tube

with a little *benzene*. When the water has settled sufficiently, read the column, allowing for the meniscus, and calculate the percentage.



Courtesy of Ind. Eng. Chem. 1926, **18**, *1348*

FIG. 111. Kingman Moisture Apparatus.

FREE FATTY ACIDS

Method of the American Chemical Society Committee.[76] REAGENTS. *Ethanol*, 95%. Freshly distilled from NaOH which gives with phenolphthalein a definite and distinct end-point.

PROCESS. Weigh into an Erlenmeyer flask 1 to 15 g. of the prepared sample. (Use a smaller amount if dark in color or high in acid.) Add 50 to 100 ml. of the hot *neutral ethanol* and titrate with stronger or weaker *standard sodium hydroxide solution* according to the degree of acidity, using *phenolphthalein indicator*.

Express results as percentage of oleic acid, except for palm oil, which may also be expressed as palmitic acid, and cocoanut and palm kernel oils, which may be expressed as both lauric and oleic acids. In reporting, state clearly the acids the figures represent.

For fats with more than 0.1% of soluble mineral matter, add to the percentage of free fatty acids as determined 10 times the percentage of bases in the soluble mineral matter as determined; this gives the equivalent of fatty acids combined with the soluble mineral matter.

SOLUBLE FATTY ACIDS

The percentage of soluble fatty acids in terms of butyric acid parallels the Reichert-Meissl number, but is less conveniently determined.

PROCESS. *Fatty Acids Separation.* To the neutralized solution obtained in the titration for the determination of the saponification number above, add carefully from the buret a volume of *0.5 N hydrochloric acid* equal to the difference between that used in the blank and that used in the actual analysis plus 1 ml. Heat on the steam bath until the fatty acids form a clear layer above the aqueous liquid, fill the flask to the neck with boiling water, and cool in ice water until the fatty acids are well hardened. Filter into a 1.5 liter flask. Repeat the addition of hot water, cooling and filtering three times. Reserve the insoluble fatty acids on the paper and adhering to the flask for the determination of insoluble fatty acids below.

Soluble Fatty Acids Titration. Titrate the combined filtrate and washings with *0.1 N potassium hydroxide*, using *phenolphthalein indicator*.

CALCULATION. Employ the following formula for the calculation of the percentage of soluble fatty acids (S):

in which T is the milliliters of 0.1 N alkali used in the titration, 5 is the equivalent of the 1 ml. of 0.5 N acid added in the determination of the saponification number, 0.0088 is the weight in grams of butyric acid equivalent to 1 ml. of 0.1 N alkali, and G is the grams used for the analysis.

Insoluble Fatty Acids

(Hehner Number)

The Hehner number is the percentage of insoluble fatty acids in the oil or fat.

Hehner Gravimetric Method. As first proposed by Hehner,[77] the pioneer English food analyst, a separate portion of 5 g. was saponified and titrated. As here described, the insoluble fatty acids separated in the foregoing operation are used.

Process. Drain the cake of insoluble fatty acids in the flask and on the paper and dry for 12 hours. Transfer the cake and as much as possible of the insoluble fatty acids on the paper to a weighed dish or wide-mouth flask. Dissolve any of the acids remaining in the flask in hot *absolute ethanol* and pour through the filter into the weighed dish or flask. Repeat the treatment until all the acids are removed, then evaporate the ethanol, dry for 2-hour periods at 100° to constant weight.

Calculation. Obtain the Hehner number by multiplying the weight in grams by 100 and dividing by the weight of the charge.

Examples. The Hehner number of common edible oils (olive, cottonseed, sesame, mustard, rape, peanut, maize, soy, linseed, etc.) ranges from 93 to 97, of butter from 86.5 to 90, of cocoanut fat 88.5 to 90.5, of palm kernel fat from 91 to 91.5, and of beef and mutton tallow from 95 to 96. The number for cacao butter is about 94.5.

Solid Fatty Acids

Twitchell Lead Acetate Gravimetric Method.[78] Solid fatty acids are precipitated by lead acetate in alcoholic solution instead of by the usual lead salts-ether method.

Process. *Precipitation of Solid Acids as Lead Salts.* Weigh into a beaker a quantity of the fatty acids, calculated to contain 1 to 1.5 g. of solid acids, separated from the sample by the usual process. (See modifications below.) This quantity will be about 10 g. if the sample is a very liquid oil, but only 2 or 3 g. if a tallow. Dissolve in *ethanol* and heat to boiling. Dissolve 1.5 g. of *normal lead acetate* [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] in a separate beaker in *ethanol*, adjust the total ethanol in the two solutions to about 100 ml., heat to boiling, and add to the hot solution of the fatty acids. Allow to cool to room temperature, then hold at about 15° overnight, filter, and test the filtrate for lead with a few drops of *ethanol-sulfuric acid mixture;* if there is no lead present, the process must be repeated, less fatty acid or more lead acetate being used.

Reprecipitation. To remove the contamination of lead salts of liquid acids, wash the precipitate with *ethanol* until a portion of the filtrate diluted with water remains clear, then transfer the precipitate back to the beaker by means of about 100 ml. of *ethanol*. Add 0.5 g. of *acetic acid* and heat to boiling, continuing the boiling until the precipitate dissolves. Cool to room temperature, hold overnight at about 15°, filter, and wash with *ethanol* as before. By this reprecipitation practically pure lead salts of the solid acids are obtained.

Solid Acids Liberation and Extraction. Transfer the precipitate to the beaker, this time by means of *ether*. Add *dilute nitric acid* sufficient to decompose the lead salts, pour into a separatory funnel, wash, and shake. Draw off the aqueous layer, wash the ether solution with water until the washings are neutral to *methyl orange indicator*. Evaporate the ether solution of the solid fatty acids in a weighed dish, dry in an oven at 110°, and weigh.

EXAMPLES. The following percentages and iodine numbers respectively of the solid fatty acids are given by Twitchell: cottonseed oil 23.13 and 0.72, soy bean oil 17.01 and 0.85, olive oil 10.93 and 1.75, peanut oil 16.58 and 0.80, lard 40.02 and 0.58, tallow 53.62 and 4.38, partly hydrogenated cottonseed oil 50.62 and 42.21. The solid acids of the last sample were redissolved and the acids reprecipitated, but the iodine number was reduced only to 40.07.

I. Baughman and Jamieson Modification.[79] The distinctive feature of the modification is the use of the original fat instead of the insoluble fatty acids; this shortens the process and facilitates the analysis of hydrogenated oils.

PROCESS. *Saponification.* Weigh into a 300-ml. Erlenmeyer flask an amount of the sample (never over 6 g.) that is estimated to contain 1 to 1.5 g. of solid acids. Saponify in the usual manner with an excess of *4% ethanolic potassium hydroxide solution*, 40 ml. being sufficient for 6 g. of fat. Neutralize to *phenolphthalein* the excess of alkali with *glacial acetic acid*, add one drop in excess, and dilute with *ethanol* to 150 ml.

Lead Salts Precipitation. Heat to boiling and pour in 5 g. of *normal lead acetate* in 50 ml. of *ethanol*, also heated to boiling. Allow to cool slowly, first to room temperature and then overnight in a refrigerator at about 15°. Filter through an 11-cm. paper and wash the flask and precipitate with cold *ethanol* until the washings, on dilution with water, remain clear. Wash the precipitate back into the flask, using about 100 ml. of *ethanol*. If the filtrate on testing with *sulfuric acid* contains no lead, start anew, using less fat.

Reprecipitation. Add 0.5 ml. of *glacial acetic acid* and boil until the precipitate dissolves. Cool slowly in the room and then overnight in a refrigerator, filter, and wash as before.

Solid Acids Liberation and Extraction. Transfer the precipitate to a 500-ml. separatory funnel by means of *ether*, add 25 ml. of *1 + 1 hydrochloric acid*, first to the flask and then to the separatory funnel, rotating to aid in the decomposition of the lead salts. Draw off the acid layer and wash the ethereal solution with several 50-ml. portions of water until the washings give no turbidity with *silver nitrate solution*. Dehydrate the ethereal solution with 6 g. of *anhydrous sodium sulfate* and pour the solution through a 7-cm. paper into a tared 200-ml. Erlenmeyer flask. Wash the flask, sodium sulfate, and filter paper repeatedly with *ether*, distil the combined ether extract and washings, and dry the residue at 110° (in an atmosphere of *carbon dioxide* if the acids contain isoöleic acid from dehydrogenated fat) to constant weight.

II. Procter and Gamble Modification.[80] As used in the Procter and Gamble Laboratories and adopted by the Committee on Analysis of Commercial Fats and Oils of the American Chemical Society, the modification is as follows.

PROCESS. *Total Acids Precipitation.* Weigh about 25 g. of the melted sample into a 600-ml. beaker. Add about 15 g. of *potassium hydroxide*, dissolved in a small amount of water, and 25 ml. of *ethanol*, and evaporate on a steam bath or hot plate (avoiding burning) to dryness. Add 200 ml. of water, heat on the steam bath until the soap has dissolved, then add *hydrochloric acid* with stirring until the soap is completely broken up and the solution is acid to *litmus paper*. Heat the solution containing the curds of fatty acids on the steam bath or hot plate until the mixture is mobile and pour into a 500-ml. separatory funnel, using 100 to 150 ml. of *ether* to aid in the transfer. Shake thoroughly, remove the aqueous layer, and wash the ether solution 3 times or until free from acid, as indicated by *methyl orange*, draining off the water solution as completely as possible. Finally remove all traces of ether by heating in a Soxhlet flask on a steam bath in a current of inert gas and weigh.

Solid Acids Precipitation as Lead Salts. Weigh into a 250-ml. beaker a quantity (not exceeding 5 g.) of the mixed fatty acids equivalent to 1.2 ± 0.3 g. of solid fatty acids. Into another beaker weigh 1.5 g. of *powdered normal lead acetate.* Add to each beaker 50 ml. of *ethanol*, cover with a watch glass, bring to a boil, and pour the lead acetate solution into the fatty acids solution, stirring continually with a glass rod which is left in the liquid, thus precipitating the solid acids. Cool to room temperature in an ice bath at 15° for 2 hours or in a refrigerator at 15° overnight. Filter through a 7.5-cm. Büchner funnel, using 200 ml. of *ethanol* cooled to 15° to transfer the precipitate from the beaker and wash on the funnel. If a few drops of *sulfuric acid* added to 50 ml. of the filtrate fail to show an excess of lead, start anew, using a smaller amount of fatty acids.

Reprecipitation. Transfer the lead precipitate back to the original beaker with the aid of 100 ml. of warm *ethanol,* add *glacial acetic acid* equivalent to 0.5% of the solution, and heat to boiling with stirring to assure complete solution of the lead salts. Cool to room temperature, then to 15° in ice water for 2 hours or in a refrigerator overnight, filter on a Büchner funnel, and wash as before.

Solid Acids Liberation and Extraction. Transfer to the original beaker, using this time about 75 ml. of *ether*, add 20 ml. of *1 + 3 nitric acid* to break up the lead soaps, and pour into a 500-ml. separatory funnel with the aid of *ether.* Remove the last traces of the precipitate from the beaker by 5 ml. additional of *1 + 3 nitric acid* and add to the main portion in the separatory funnel. Shake and draw off the aqueous layer, then wash the ether solution with water until neutral to *methyl orange.* Finally transfer the ether solution to a tared 150-ml. Soxhlet flask, evaporate the ether in a current of inert gas, dry in an oven at 103° for 1 hour, cover, cool, and weigh, repeating the heating until the weight is constant.

CALCULATION. Obtain the percentage of solid fatty acids by multiplying the weight of the dried precipitate by 100 and dividing by the weight in grams of the total fatty acids taken.

III. Cocks, Christian, and Harding Modification.[81] Although Cocks *et al.* obtained results by the Twitchell method on natural oils agreeing within about 1%. on partially hydrogenated whale and soy oils the discrepancy reached about 2% for saturated and about 3% for solid unsaturated acids, owing to the solubility of the lead salts in ethanol.

PROCESS. *Solid Acids Precipitation as Lead Salts.* Dissolve 3.5 g. of freshly prepared fatty acids and 3.45 g. (1 g. if there is less than 25% of solid acids in the sample) of *normal lead acetate* [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$], each in 50 ml. of *92 to 93% (by wt.) ethanol.* Heat both solutions to boiling and pour the second into the first. Mix, heat again to boiling, cool slowly, and allow to stand overnight at 15 to 20°. Next morning stir the mixture, filter from the ethanol on a 10-cm. Büchner funnel, and, when the ethanolic solution has passed through, transfer the funnel to a clean filter flask. Wash the lead salts adhering to the precipitating vessel and on the funnel with 100 ml. of *naphtha* (b.p. 40 to 60°) in 20-ml. portions. Remove the naphtha from the washings by distillation. Dissolve the residue by boiling under a reflux condenser with 20 ml. of *92 to 93% ethanol* containing 1 drop of *glacial acetic acid*, and allow the lead salts of the solid acids to crystallize at 15 to 20° for 3 hours, then filter, wash with 20 ml. of cold *92 to 93% ethanol*, and unite with the main precipitate left in the Büchner funnel after washing with *naphtha.*

Solid Fatty Acids Liberation. Decompose the lead salts with *dilute nitric acid* and shake with *methylated ether.* Wash the latter, containing the total solid acids, free from mineral acid and transfer to a weighed dish. Weigh

the total solid fatty acids and use the whole or part for the determination of the iodine number.

CALCULATION. Obtain the amounts of solid saturated and solid unsaturated acids, assuming that the latter has a mean iodine number of 90 unless there is evidence to the contrary (e.g., presence of erucic acid from rape oil with an iodine number of 75.5). Calculate the percentages of both the saturated and the unsaturated solid acids.

Bertram Permanganate Gravimetric Method.[82] The method depends on (1) splitting the double bond of oleic, linoleic, and other unsaturated liquid acids by oxidation with permanganate with the formation of oxy acids and other derivatives insoluble in naphtha, and (2) the removal of lower saturated acids, taking advantage of the solubility of their magnesium salts.

PROCESS. *Saponification.* Saponify 5 g. of the sample in a 300-ml. Erlenmeyer flask by refluxing for 1 hour with 75 ml. of *0.5 N ethanolic potassium hydroxide solution*, cool, and titrate back with *0.5 N hydrochloric acid*. Make a blank determination and obtain the saponification number by difference.

Unsaponifiable Matter Separation. Transfer the soap solution to a separatory funnel, rinsing with 20 ml. of *0.5 N ethanolic potassium hydroxide solution* and 75 ml. of water. Shake out 3 times with *naphtha* (b. p. 40 to 60°), wash the combined extracts once with *dilute potassium hydroxide solution*, and twice with water, then add the wash liquid to the soap solution. Evaporate the naphtha solution and dry in a boiling water oven. Multiply the weight of the residue by 20 to obtain the percentage of unsaponifiable matter. As noted below, Hilditch and Priestman do not remove the unsaponifiable matter prior to oxidation.

Unsaturated Acids Oxidation. Evaporate the soap solution on the steam bath to remove the ethanol, heating cautiously over a free flame to drive off the last traces, then wash into a 2-liter flask, cool, add 5 ml. of *potassium hydroxide solution*, and warm until clear. Cool and add an excess of *4.5% potassium permanganate solution*, keeping the temperature below 25° (see Hilditch and Priestman below); add more *permanganate solution* if the solution loses its violet-red color. Shake thoroughly and allow to stand overnight. Decolorize with *dilute sulfuric acid* and *concentrated sodium bisulfite solution*, warming (but not boiling) until the manganese dioxide dissolves and the fatty acids separate as a clear layer.

Naphtha Extraction. Shake out the mixture with *naphtha* and wash with 3 portions of water, then evaporate the naphtha solution.

Saturated (Solid) Acids Precipitation with Magnesium Sulfate. Dissolve the residue in 200 ml. of water, make slightly alkaline with *ammonium hydroxide*, add 30 ml. of *10% ammonium chloride solution*, heat to boiling, then add an excess of *15% magnesium sulfate solution* and heat again to boiling, thus precipitating the higher acids. Cool, filter through a Gooch crucible or other asbestos filter, and wash. Transfer the filter and precipitate to an Erlenmeyer flask with *dilute sulfuric acid*, dissolve, and repeat the precipitation with *ammonium hydroxide, 10% ammonium chloride solution*, and *15% magnesium sulfate solution*, then again filter and wash.

Solid Acids Extraction. Boil the filter and precipitate with *dilute sulfuric acid* under a reflux condenser, cool, shake out twice with *naphtha*, wash the extract with water, evaporate to dryness, and dry the residue of higher solid fatty acids to constant weight in a boiling water oven.

CALCULATION. Multiply the weight of the higher solid fatty acids by 20 to obtain their percentage in the sample.

I. Hilditch and Priestman Modification.[83] After an exhaustive study of the Bertram permanganate method and the Twitchell lead

salt method, Hilditch and Priestman recognize the faults of both, but on the whole prefer the Bertram method. They do not consider it necessary to separate the unsaponifiable matter prior to oxidation but consider that it is highly important to carry out the oxidation at between 35 and 40°, not below 25° as recommended by Bertram, nor above 50°.

II. Grossfeld Modification.[84] This modification, designed for the determination of lauric acid and its higher homologs, differs from Bertram's original method as follows: (*a*) only 500 mg. of fat are used, (*b*) the reaction time is only 30 minutes if lauric acid is present, 60 minutes if absent, (*c*) the oxidation is at 20°, (*d*) the fatty acids are separated by filtration in the presence of *dilute ethanol* followed by extraction of the filter with *naphtha*, instead of shaking with naphtha at the start, and (*e*) nonylic acid is determined, after precipitation with magnesium sulfate, by distillation of a portion of the supernatant liquid with *phosphoric acid* to half volume, thus obtaining in the distillate 99.5% of the nonylic acid. On treatment with *alkaline permanganate solution*, lauric, myristic, and palmitic acids are partially oxidized, the extent being in descending order.

Saturated and Unsaturated Higher Fatty Acids

Gusserow-Varrentrapp Lead Salt-Ether Gravimetric Method. Lewkowitsch[85] has pointed out that the separation of insoluble solid from insoluble liquid acids by the original method[86] is not a separation of saturated from unsaturated acids and furthermore that unsaturated and liquid fatty acids are not coterminous, although often so used in the literature. He lists fatty acids according to the deportment of their lead salts with ether as (1) *insoluble* (palmitic, stearic, arachidic, behenic), (2) *sparingly soluble* in the cold (erucic, petroselinic, isoöleic), and (3) *easily soluble*, especially with heat (oleic, linoleic, linolenic, clupanodonic).

Baughman and Jamieson Modification.[87] The procedure, as noted in the authors' description, is not suited for butter fat and coconut and palm kernel oils containing saturated acids with carbon atoms below 14, nor for those containing the unsaturated acids erucic, chaulmoogric, hydnocarpic, and isoöleic acids. Nevertheless if the details outlined by them are followed, agreeing results may be obtained.

Process. The somewhat lengthy details are readily carried out in four distinct steps as follows.

1. *Saponification, Neutralization, and Precipitation of Lead Salts of Fatty Acids.* Saponify 10 g. of the sample by heating for 30 minutes on a steam bath with 30 ml. of *ethanol* and 8 ml. of *50 + 50 potassium hydroxide solution.* Slightly acidify with *1 + 2 acetic acid*, using *phenolphthalein indicator*, then add with rotating *3 + 20 potassium hydroxide solution* to a distinct pink. Add the neutralized soap solution to a boiling mixture of 60 ml. of *20% lead acetate solution* and 60 ml. of water, rinsing with 5 ml. of *ethanol*, and a little hot water. Boil gently for 5 minutes, shake well, and cool in a stream of water while rotating the flask to distribute the lead soap over the interior surface. Cool, pour off the aqueous solution into a large beaker and examine for turbidity which should be due solely to basic acetate and not to particles or globules of lead soap. Wash the lead soap in the flask twice with cold water, drain for 10 minutes, and remove the drops of water by absorption on filter paper held in forceps, avoiding strong pressure.

2. *Separation of Lead Salts of Soluble Unsaturated Acids from Those of Insoluble Saturated Acids.* Shake by rotation the soap deposit in the flask with 120 ml. of *ether* for 5 minutes, reflux until the soap is well disintegrated, rinse down the sides of the flask with *ether* until the final volume is about 150

ml., cover with a beaker, and cool in a refrigerator overnight. Fit a 7-cm. ordinary filter paper in a 7.5-cm. Büchner funnel with suction, then carefully fit an 8-cm. hardened paper, and decant the ether solution from the flask onto the funnel, avoiding suction sufficiently rapid to cause clogging with soap or ice. Wash the precipitate into the filter with small portions of *ether*, keeping the funnel covered in the interim and closing with a spoon any cracks that form in the soap; then wash with 8 to 10 portions of *ether*, finally allowing the mass to crack into pieces.

3. *Liberation, Extraction, and Determination of Saturated Acids.* Immediately after the insoluble soap falls into pieces, remove with a spoon to a 500-ml. separatory funnel, using about 50 ml. of *ether* to wash off the paper, shake to break up lumps, allow to stand 20 minutes, then add 20 ml. of *hydrochloric acid* diluted with 10 ml. of water, and shake again 2 minutes. Place the paper with any adhering soap in the liter flask used for the precipitation, add 5 to 10 ml. of *dilute hydrochloric acid*, shake well, and rinse into the separatory funnel with alternate small portions of *ether* and water.

Rotate the separatory funnel and, after allowing to stand 10 minutes, draw off slowly and reject the aqueous layer from the ether layer and any emulsion or floating lumps of undecomposed lead soap. If the latter is present, add a little *diluted hydrochloric acid*, then 20 ml. of water, shaking after each addition, allow to stand, remove and reject the aqueous solution as before. Wash the ether solution with 25-ml. portions of water until all the acid is removed, dehydrate with 2 g. of *anhydrous sodium sulfate*, and transfer to a tared 300-ml. Erlenmeyer flask, rinsing with small portions of *ether* and avoiding transfer of any sodium sulfate.

Distil off the ether, dry the saturated acids at 110° to constant weight, and calculate the percentage of impure saturated acids. Determine the iodine number of the impure saturated acids and correct the percentage as directed below.

4. *Liberation, Extraction, and Determination of Unsaturated Acids.* Pour the ether solution of the soluble lead soap obtained as directed above into a 500-ml. separatory funnel, rinse the filter flask with a little *ether*, add 30 ml. of *hydrochloric acid* diluted with 75 ml. of water, and shake by rotating for 2 minutes. Allow to stand for 10 minutes and draw off the aqueous solution into a beaker. If drops of the ether solution are carried through with the lead chloride precipitate, decant the solution from the precipitate back into the separatory funnel, rinsing with a little *ether*, rotate, let stand for 10 minutes, draw off and reject the aqueous solution, and wash the ether solution with 50-ml. portions of water until all acid is removed. Pour the ether solution into a 300-ml. weighed Erlenmeyer flask, rinsing with a little *ether*, remove the ether by distillation, and dry at 110° for 1 hour in a stream of *carbon dioxide*, continuing the flow during cooling. Repeat the drying and cooling until the weight is constant.

CALCULATION. Calculate the percentage of impure saturated acids. Determine the iodine number and correct.

Corrections. The results are corrected for unsaturated acids in the saturated acid fraction by the following formulas:

$$A = \frac{IS \times 100}{IU}$$

$$C = \frac{A \times B}{100}$$

in which A is per cent of unsaturated acids in impure saturated acids fraction, B is per cent of impure saturated acid as above determined, IS and IU are iodine number of impure saturated and unsaturated acids respectively determined in the fractions after weighing, C is correction to be deducted from the per cent of impure saturated acids

and added to the per cent of impure unsaturated acids calculated from the weight.

Stearic Acid

Hehner and Richmond Method, Modified by Holland, Reed, and Buckley.[88] In the process as devised by Hehner and Richmond,[89] an ethanolic solution of pure stearic acid saturated at 0° is employed as a solvent for the fatty acids other than stearic. For the purpose of obviating the error due to supersaturation, a solution containing an excess of stearic acid over that dissolved at 0° is substituted by Holland *et al*. for the original solution and a correction is introduced. The modified method gave 7 to 22% of stearic acid in butter, whereas earlier investigators found little or none.

PROCESS. Weigh out 0.5 g. of the melted insoluble acids into an 8-ounce sterilizer bottle, add 150 ml. of *0.3% stearic acid solution in ethanol* at 30° from a pipet, close with a rubber stopper, shake while gradually increasing the temperature until the solution clears up, then place in a pocket of an ice tank, and allow to stand overnight. Carefully invert the bottle several times and some hours after suck off the liquid as completely as possible through a small thistle tube covered with a piece of muslin. Dissolve the residue in *ether*, transfer to a tared flask, distil off the ether, dry at 100°, and weigh.

Blank. Conduct a blank determination on a weighed amount of stearic acid equivalent to that expected in the actual analysis, and deduct that weight from the weight found, thus obtaining the true blank to be used in correcting the result of the actual analysis.

Palmitic Acid

Determine by difference. If a fat consists entirely of triolein, tristearin, and tripalmitin, calculate the oleic acid from the iodine number, the stearic acid by the foregoing method, and the palmitic acid by subtracting the sum of the oleic and stearic acids from the total fatty acids.

Oleic Acid

In the absence of other unsaturated acids, the percentage of oleic acid in the insoluble fatty acids (O) may be calculated from the iodine number of these acids (I) and the iodine number of pure oleic acid (90.07) by the following formula:

$$O = \frac{100I}{90.07}$$

The percentage of liquid insoluble acids, as determined by the lead soap method of Varrentrapp,[90] in many cases is the approximate percentage of oleic acid. The method involves saponification, precipitation with *lead acetate*, and separation of the soaps of the liquid and solid acids by ether essentially as described for the determination of arachidic acid below under Peanut Oil.

By shaking the ether solution, containing the lead oleate, with *10% hydrochloric acid*, the free oleic acid is liberated and may be obtained quantitatively by evaporating the ether solution, after washing with water, drying, and weighing.

If the residue is pure oleic acid, it will have an iodine number of 90.07. See also formulas under Thiocyanogen Number above.

Glycerol

Smith and Duke Perchlorato Cerate Volumetric Method.[91] The reaction on which the method (University of Illinois) depends follows:

$$C_3H_8O_3 + 8H_2Ce(ClO_4)_6 + 3H_2O$$
$$= 3HCOOH + 8Ce(ClO_4)_3 + 24HClO_4$$

The back titration is with standard sodium oxalate, using nitro-ferroin as indicator.

REAGENTS. *Standard Ammonium Perchlorato Cerate Reagent.* Dissolve 55 or 56 g. of $(NH_4)_2Ce(NO_3)_6$ in 340 to 345 ml. of 72% $HClO_4$, stir well for a half minute, then add 100 ml. of water. Repeat the stirring and addition of 100 ml. of water until the solution measures 1 liter. Store in glass-stoppered bottles in the dark. Avoid carbon and dust. Standardize every fortnight against 0.1 N $Na_2C_2O_4$ solution, using nitro-ferroin as indicator.[92]

Sulfato Cerate Solution. Dissolve 63 to 65 g. of $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ in 1 liter of 0.5 M H_2SO_4. Dark storage is unnecessary.

Standard Sodium Oxalate Solution. Dissolve 13.412 g. of the Bureau of Standards reference salt per liter of 0.1 normal $HClO_4$.

Ferrous Sulfate Reagent. Dissolve 40 g. of Mohr's $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ in 1 liter of 0.5 N H_2SO_4. Store under hydrogen and standardize against perchlorato cerate solution, using ferroin as indicator.

Ferroin (o-Phenalthroline) and Nitro-Ferroin (Nitro-o-Phenanthroline). Obtainable in solution form.

Potassium Hydroxide Solution, 0.5 N ethanolic. Dissolve 16 g. of KOH in the least required amount of water and dilute to 1 liter with ethanol. Allow to settle 2 or 3 days and decant.

PROCESS. *Saponification.* Heat the sample at 80°, filter, decant from water, and reflux 10 g. with 100 ml. of *0.5 N ethanolic potassium hydroxide solution* for 90 minutes. Transfer the solution to a beaker, rinse with hot water, dilute to 350 ml., then evaporate by cautious boiling to about 50 ml. Dilute to 250 ml. and reevaporate to 50 ml.

Perchloric Acid Treatment. Add to the soap solution 20 ml. of *72% perchloric acid diluted to 100 ml.*, and heat on the steam bath until fatty acids separate. Filter to remove the fatty acid cake and precipitated potassium perchlorate and wash with cold water.

Protein Removal. To the filtrate, add *basic lead acetate solution.* If a precipitate forms, add more stepwise, avoiding a large excess. Filter, evaporate, transfer to a 500-ml. flask, and dilute to volume. Repeat the procedure, omitting the sample, as part of a blank determination.

Perchlorato Cerate Treatment. Pipet 10-ml. aliquots of the deproteinized solution and blank to 400-ml. beakers. Add from a pipet *standard perchlorato cerate solution* in sufficient excess, dilute to 100 ml. with *4 formal perchloric acid,* warm on the steam bath to 50°, and hold at that temperature for 15 minutes. At no time should the temperature reach 60°.

Titration. Cool the solution and titrate the excess of perchlorato cerate ion with *standard sodium oxalate solution,* using 2 drops of *0.025 M nitro-ferroin indicator.*

CALCULATION. Obtain the per cent of glycerol (G) by the following formula:

$$G = 92M(C - N)/(80W \times E)$$

in which C is the milliliters of cerate solution taken multiplied by its standard factor, N is the milliliters of oxalate solution required multiplied by its standard factor, M is the standard dilution of sample in milliliters, W is the weight of sample, and S is the milliliters of aliquot taken.

NOTE. If the use of perchlorato cerate standard solution and perchloric acid is objectionable, the usual Procter and Gamble procedure[93] may be employed, with the substitution of a sulfuric acid solution for the perchloric acid solution of the glycerol and the use of the sulfato cerate solution in sulfuric acid as the oxidant. This necessitates heating the sample and excess of oxidant to 90 to 100° for 90 minutes before back-titration, and using standard ferrous sulfate to determine the excess of sulfato cerate, with ferroin instead of nitro-ferroin as indicator.

UNSAPONIFIABLE MATTER

American Chemical Society Committee Gravimetric Method.[94] APPARATUS.

Stoppered Extraction Cylinder. About 35 mm. in diameter and 30 cm. high, graduated at 40, 80, and 130 ml.

PROCESS. *Saponification.* Place 5 g. (±0.2 g.) of the carefully prepared sample, 30 ml. of *redistilled ethanol*, and 5 ml. of *50% potassium hydroxide solution* in a 200-ml. Erlenmeyer flask, attach to a reflux condenser, and boil for 1 hour.

Naphtha Extraction. Pour the soap solution into the extraction cylinder and wash to the 40-ml. mark with the *ethanol*, then complete the transfer, using first warm and later cold water to the 80-ml. mark, and finally a small amount of *redistilled naphtha* (b.p. below 75°). After cooling to room temperature, add 50 ml. of *naphtha*, shake vigorously for 1 minute, and allow to settle until both layers are clear. Using a slender glass siphon, draw over the naphtha layer, measuring about 40 ml., into a 500-ml. separatory funnel, repeating the extraction with 50 ml. of *naphtha* 6 times or more. Wash the combined solutions 3 times by shaking vigorously with 3 portions of 25 ml. each of *10% ethanol.* Draw off the naphtha solution into a tared beaker or wide-mouth flask, evaporate off the naphtha on a steam bath in a current of air, and dry as directed for the determination of Moisture and Volatile Matter above. Weigh and correct for the amount obtained in the blank determination as described below.

Test the unsaponifiable matter for solubility in 50 ml. of *naphtha* at room temperature. If any insoluble matter remains, filter, wash, and evaporate the extract. Dry and weigh as before. Thorough and vigorous shaking, to insure intimate contact of the two phases, is emphasized.

Blank Determination. Evaporate 25 ml. of the redistilled *naphtha* with about 0.25 g. of stearin or other hard fat, drying as in the actual analysis. The blank should yield only a few milligrams of material.

Paraffin, Mineral Oil, and Resins. If added to animal or vegetable oils, they are detected by their high content of unsaponifiable matter and physical tests. Rosin oil shows a high polarization, whereas edible oils seldom exceed one degree on the sugar scale.

STEROLS, HYDROCARBONS, AND TOTAL UNSAPONIFIABLE MATTER

Grossfeld Dual Determination Gravimetric Method.[95] Two determinations of unsaponifiable matter are made with ratios of fat to solvent of 1 : 10 and 1 : 80 respectively. The calculation of sterols and hydrocarbons depends on the different amounts of the unsaponifiable matter extracted.

PROCESS A. Saponify 5 g. of the sample in a 100-ml. flask with 20 ml. of *ethanol* and 3 ml. of *47% sodium hydroxide solution.* Cool to 30 to 40°, add 50 ml. of *naphtha* (b.p. 60 to 70°), stopper, shake several times, then add 20 ml. of water, again stopper, shake 30 times, and let stand overnight. Evaporate a 25-ml. aliquot of the naphtha solution in a tared Erlenmeyer flask, dry 1 hour at 105°, weigh, and calculate the per cent of the fraction of unsaponifiable matter extracted (A) in terms of per cent of the fat by the factor 35.

PROCESS B. Saponify 2.5 g. of the sample with 20 ml. of *ethanol* and 1 ml. of *47% sodium hydroxide solution* for 5 minutes. Cool to 30 to 40°, dissolve in 200 ml. of *naphtha*, and treat as under method A, but using 12.5 (not 20) ml. of water. Pipet 150 ml. of the solvent solution into a flask, add 20 ml. of *0.01 N sodium hydroxide solution*, shake well, and let stand overnight. Remove an aliquot of 100 ml. of naphtha solution to an Erlenmeyer flask, distil, dry the residue 1 hour at 105°, and weigh. As a check dissolve the residue from the naphtha and weigh the flask. Calculate the content of unsaponifiable matter thus extracted (B) by the factor 73.4.

CALCULATION. Obtain the percentage of sterols (S), hydrocarbons (H), and total

unsaponifiable matter $(S + H)$ by the following formulas:

$$S = 100 \times \frac{B - A}{N - M}$$

$$H = A - \frac{M(B - A)}{N - M}$$

$$S + H = \frac{B(100 - M) - A(100 - N)}{N - M}$$

in which A and B are as above given and N and M are average constants which, as found by Grossfeld, are 74 and 26 for butter fat, whale oil, linseed oil, peanut oil, cacao butter, and lard, and 82 and 44 for cocoanut oil respectively. Applying these constants, the formulas reduce to the following:

GENERAL FORMULAS

$$S = 2.08(B - A)$$
$$H = 1.54A - 0.54B$$
$$S + H = 1.54B - 0.54A$$

FORMULAS FOR COCOANUT OIL

$$S = 2.63(B - A)$$
$$H = 2.16A - 1.16B$$
$$S + H = 1.42B - 0.47A$$

PHYTOSTEROL AND CHOLESTEROL

Bömer Acetate Test.[96] Bömer crystallized the sterols from the unsaponifiable matter prepared in the usual manner and distinguished cholesterol from phytosterol by the forms of the crystals. Later the same author[97] converted the sterols into acetates by heating with *acetic anhydride* and determined the melting point, after repeated recrystallization, as a means of detecting vegetable fatty matter in animal fats. The melting point of cholesterol acetate was 114.3 to 114.8° and of phytosterol acetate 125.6 to 137.0°. The wide range of the latter points to the presence of vegetable sterols other than phytosterol. A melting point above 116° was looked on with suspicion and above 117° as proof of the presence of vegetable fat.

Ethanol Extraction Modification. This process, adopted by the Bureau of Animal Industry[98] and the Association of Official Agricultural Chemists,[99] is particularly useful in detecting vegetable fats in butter, lard, and their substitutes, but is not applicable in the presence of hydrogenated soy bean oil.

APPARATUS. *Extraction Assembly.* A flat-bottom liter flask connected with a reflux condenser and a 700-ml. round-bottom flask that delivers ethanol vapor through a bent tube extending to the bottom of the liter flask into the melted fat. A siphon tube, provided with a pinchcock, passing through a third hole in the stopper of the liter flask, is adjusted to draw off the condensed ethanol from the melted fat layer into the 700-ml. flask.

PROCESS. *Saponification.* Heat 250 g. of the sample, contained in the flat-bottom liter flask, on a steam bath and pass the vapor from 500 ml. of boiling *ethanol* from the round-bottom flask into the melted fat. When all the ethanol has passed into the liter flask, disconnect the inflow tube and, by opening the pinchcock, siphon the ethanol layer back into the 700-ml. flask. Repeat the extraction twice, evaporate the ethanol extract to about 250 ml., then without cooling add 20 ml. of a *1 + 1 potassium hydroxide solution,* saponify by boiling 10 minutes, and cool.

Ether Extraction. Transfer the soap solution to a 2-liter separatory funnel containing 500 ml. of *ether.* Shake well, add 500 ml. of water, mix thoroughly by rotating, but avoid an emulsion, and allow to stand 10 minutes. Draw off the aqueous layer, wash the ether solution by shaking with three 150-ml. portions of *1.1% potassium hydroxide solution* and three 150-ml. portions of water, and dry with 5 g. of *anhydrous sodium sulfate* for 30 minutes. Transfer the ether solution to a

flask and distil the ether until only 20 ml. remain, completing the distillation in a 50-ml. Erlenmeyer flask.

Acetylation. Dry at 105° for 1 hour, add 3 ml. of *acetic anhydride*, cover with a crucible, and boil gently for 30 minutes. Cool, add 30 to 35 ml. of *60% ethanol*, mix well, filter, and wash the precipitate four or five times with hot *80% ethanol*.

Crystallization. Allow the crystals of sterol acetates to separate from the filtrate kept for several hours in a bath or refrigerator at about 10°. Filter, wash the crystals with cold *80% ethanol*, then dissolve in the least possible amount of hot *absolute ethanol*, collecting the solution in a small beaker.

Recrystallization. Add 2 drops of water, warm if necessary to secure a clear solution, and allow the crystals to form, stirring to prevent accumulation on the sides. Collect the crystals on a hardened filter, wash twice with cold *90% ethanol*, and dry for 30 minutes at 100°.

Determine the melting point of the first crop of crystals, also of those formed by dissolving in *absolute ethanol* and recrystallization. Compare with the melting points given above.

Windaus Digitonin-Acetic Anhydride Method.[100] Process. To 50 g. of the melted sample in a separatory funnel, add 20 ml. of *1% ethanolic digitonin solution*, shake vigorously for 15 minutes, allow the layers to separate, and then draw off the clear fat layer. Add 100 ml. of *ether* to the ethanolic solution, containing a bulky precipitate of sterols in combination with digitonin, shake gently, filter, wash the precipitate with *ether* until the fat is removed, dry, and acetylate with 3 ml. of *acetic anhydride*, then proceed as in the Ethanol Extraction Modification above.

Klostermann Modification.[101] As used by Kühn, Benger, and Werwerinke,[102] this test is claimed to enable the detection of as little as 1% of cottonseed oil in animal fats, notably lard and oleomargarine.

Process. To the still warm fatty acids, separated from 50 g. of the sample in the usual manner, contained in a beaker, add 25 ml. of *1% digitonin solution in ethanol* and heat slowly in a water bath at a temperature not exceeding 70° for 45 to 60 minutes with occasional stirring until the precipitate of digitonide separates. Add 15 to 20 ml. of *chloroform*, filter, using suction, wash successively with *chloroform* and with *ether* until all the fat is removed, and dry 10 minutes at 90 to 100°. Boil the dried precipitate with 3 to 5 ml. of *acetic anhydride* for 5 minutes, add while hot 4 volumes of *50% ethanol*, cool, and after 5 to 10 minutes collect the precipitate of acetate on a small paper. Wash several times with cold *50% ethanol*, remove to a crystallizing dish with *ether*, recrystallize, and determine the melting point as above described.

Notes. Others who have modified the method are Marcusson,[103] Fritzsche,[104] and Kühn *et al.*[105]

Prescher[106] states that there are two kinds of digitonin on the market, one crystalline and insoluble in water, the other amorphous and soluble in water. The former is suited for the method.

Auerbach[107] points out that the digitonide method distinguishes sharply a vegetable oil from an animal oil or fat, but no method has been devised that detects the latter in the former. The most satisfactory method is to convert the digitonides into acetates, saponify, extract the alcohols with *naphtha*, and determine the melting point. Examine also the crystals under the microscope. In the presence of animal fat, cholesterol and phytosterol crystals often occur side by side, also in some cases as cone-shaped mixed crystals.

Cholesterol

See Phytosterol and Cholesterol above.

Rappaport and Klapholz Digitonin-Ferricyanide Iodometric Method.[108] The method

(Vienna University) depends on the precipitation of the cholesterol with the glucoside digitonin and the titration of the hexose separated from the excess of the digitonin re-

Reagents. *Digitonin Solution*, 0.05%. Dissolve by warming 25 mg. of digitonin in a little ethanol in a 50-ml. volumetric flask, cool, add 16.7 ml. of water, fill to the mark with ethanol, and mix well.

Phosphate Buffer. Dissolve 84.1 g. of K_2HPO_4 and 28 g. of K_3PO_4 in water and make up to 1 liter.

Hagedorn-Jensen Zinc Sulfate-Potassium Iodide Solution. Dissolve 5 g. of KI, 10 g. of $ZnSO_4 \cdot 7H_2O$, and 50 g. of NaCl in water and dilute to 200 ml.

Process. *Digitonin Precipitation.* To 1 ml. of cholesterol solution, in a test tube with a calibration mark at 12 ml., add 3 ml. of *0.05% digitonin solution*, place in a boiling water bath, turn off the flame, and hold for 5 minutes. Cool to hand warmth and precipitate the cholesterol digitonide by adding 2 ml. of water at 50° with shaking.

Treat 3 ml. of the *0.05% digitonin solution* alone for a blank determination.

Keep both tubes (blank and unknown) overnight in a cold dark place, dilute to 12 ml. with water, mix, and filter through a 7-cm. S. and S. 597 paper (rough side in).

Hydrolysis. Add 1 ml. of *40% sulfuric acid* to 10 ml. of the filtrate and hydrolyze 2 hours in a water bath, then cool, and neutralize to 0.1 ml. of *ethanolic phenolphthalein* with *2 N sodium hydroxide solution*.

Hexose Titration. Add 4 to 5 ml. of a mixture of equal parts of *0.42% potassium ferricyanide* and *phosphate buffer*. Boil 15 minutes on a water bath, cool, add 2 ml. of *zinc sulfate-potassium iodide solution*, acidify with *hydrochloric acid*, and titrate with *standard 0.005 N sodium thiosulfate solution*, using *starch solution* as indicator.

Calculation. Subtract the milliliters of alkali used for the unknown from that used for the blank and calculate the milligrams of cholesterol in the charge. One milliliter of 0.005 N sodium thiosulfate is equivalent to 0.115 mg. of cholesterol.

Ewert Digitonin Gravimetric Method.[109] The procedure involves, first, extraction from organs and other tissues and, second, precipitation and weighing as cholesterol-digitonin. The details of the process and the somewhat involved calculation were elaborated at the University of Upsala.

Monasterio Digitonin-Silver Chromate Iodometric Micro Method.[110] The method was devised at Pisa University.

Reagent. *Acid Silver Chromate Reagent.* Dissolve 5 g. of $AgNO_3$ in 25 ml. of water and add 5 g. of $K_2Cr_2O_7$ dissolved in 50 ml. of water. Mix, wash the precipitate twice with water by centrifuging, and dissolve in 50 ml. of H_2SO_4.

Process. *Digitonin Precipitation.* To 2 ml. of the chloroform solution of the cholesterol in a small test tube, add 1 ml. of *1% digitonin in 80% ethanol* and evaporate on the water bath to about half volume; let stand 6 hours, and centrifuge. Wash the precipitate 3 times with 3 ml. of *ethanol* and remove the ethanol on the water bath.

Oxidation. Add 5 ml. of the *acid silver chromate reagent* and heat 15 minutes at 160° on the sand bath. Transfer the solution to a larger test tube and continue the oxidation 45 minutes longer, adding 5 ml. of the *reagent* if necessary to secure the characteristic green color. Transfer the solution, also 5 ml. of the *reagent* heated on the sand bath in like manner, each to a 200-ml. flask, and wash well with water.

Titration. After cooling, add 10 ml. of *10% potassium iodide solution* to the unknown and the blank and titrate with *standard 0.1 N sodium thiosulfate solution.*

Calculation. Divide by 10.48 the difference between the two titrations to obtain the weight of cholesterol.

Carotene, Vitamin A, Vitamin D

See Part I, C10.

Nickel

It is important that the nickel catalyst be completely removed from hydrogenated fat.

Torricelli Dimethylglyoxime Spot Test.[111] This semi-quantitative test is essentially as follows. Treat the fat with *hydrochloric acid*, filter, and wash with hot water. Evaporate the filtrate to dryness, take up the residue in 3 to 5 ml. of water, and precipitate the iron with *pyridine*. Centrifuge, decant the clear liquid, evaporate, ignite, take up in a little *hydrochloric acid*, and dilute to a definite volume. Add the solution to filter paper impregnated with *1% methylglyoxime solution in ethanol* and compare the intensity of the color with that obtained with known amounts.

d. Keeping Quality and Spoilage

Rancidity

Kreis Phloroglucinol Test.[112] As originally performed, 2 ml. of the oil or melted fat are shaken with 2 ml. of *hydrochloric acid* and 2 ml. of *0.1% phloroglucinol in ether*. If the fat is rancid, the red or pink color appears on standing, the depth of color being roughly proportional to the degree of rancidity. Kreis attributed the reaction to the presence of an aldehyde or ketone.

Powick[113] showed that the substance in rancid fat that gives the color is *epihydrinaldehyde*.

Pyke[114] considers that the epihydrinaldehyde is produced by the formation of a double peroxide of oleic acid followed by the breaking up of the fatty acid into heptylic aldehyde, the half aldehyde of pimelenic acid, and the oxide of acrolein-β-carboxylic acid, which itself is converted into epihydrinaldehyde.

The method as first proposed has been severely criticized, but as modified by several workers it is believed to be a valuable means of detecting rancidity.

I. Kerr Modification.[115] Process. Pipet 10 ml. of the sample into a test tube (8 x 1 in.), add 10 ml. of *hydrochloric acid*, close with a rubber stopper, and shake vigorously for 30 seconds. Add to the mixture 10 ml. of *0.1% phloroglucinol solution in ether*, stopper, and shake vigorously, then allow to stand and note the color.

If the fat is rancid, the acid layer takes on a red or pink color.

Dilute the original oil or melted fat with increasing volumes of *kerosene* and note the highest dilution at which the red or pink is evident on performing the test, ignoring orange or yellow.

II. Täufel, Sadler, and Russow Modification.[116] Place in a test tube 1 ml. of the oil or melted fat and 1 ml. of *hydrochloric acid*, taking care not to wet the sides. Introduce halfway down in the tube a wad of cotton, wet on the lower side with 1 ml. of *1% phloroglucinol in ether* and 10 drops of *20% hydrochloric acid*. Mix the oil and acid by a gentle motion, without splashing the cotton, for 1 to 2 minutes, then heat in the water bath to a temperature not exceeding 60°.

The presence of epihydrinaldehyde is indicated by a red spot in the wad.

III. Frehden Modification.[117] As performed by Frehden in conjunction with other tests noted below, equal volumes of the sample and *hydrochloric acid* are mixed in a micro crucible which is covered with a disk of filter paper moistened with a *0.1% solution of phloroglucinol in ethanol* and a few drops of *20% hydrochloric acid*. On gently heating the crucible, a distinct red coloration appears in the paper.

Frehden Diaminofluorine Test.[117] Reagent. Dissolve 0.1 g. of 2,7-diaminofluorine in 5 ml. of glacial acetic acid and add 5 mg. of hemin as a catalyzer.

PROCESS. Place a drop of the *reagent* on a piece of filter paper and add to it a little of the sample. A deep blue quinoid product will form with the peroxide present if the sample has become rancid.

Stamm Diphenylcarbazide Test.[118] REAGENT. Mix 0.1 g. of *diphenylcarbazide* with 10 ml. of vaseline oil.

PROCESS. Stir 10 drops of the oil, or a little of the melted fat mixed with *vaseline oil*, with 5 drops of the *reagent* and heat for 3 minutes. The formation of a red color indicates that the sample will soon become rancid, although at the time it may appear normal. The test is an adaption of Korpácsy's test for hydroxy aliphatic acids.

Frehden Modification.[119] Frehden dissolves 0.5 g. of *sym. diphenylcarbazide* in 100 ml. of *tetrachloroethane* and adds a drop of this reagent to a little of the solution of the oil or melted fat on a piece of filter paper.

Taffel and Revis Iodometric Method.[120] Employ methods A or B below, according as the rancidity is due to exposure of the sample to air at moderate or high temperatures respectively. Of the two procedures for method A, the first (*a*) is for samples yielding a slight color by the Kreis-Kerr test and the second (*b*) for samples yielding a strong color. For the Kreis-Kerr test, 10 drops of 5% phloroglucinol in ethanol (not ether) to 10 g. (5 g. if very rancid) of the fat are used.

Method A (Rancidity Due to Exposure to Air at Moderate Temperatures). (*a*) *Rancidity Slight.* To 10 g. of the oil or fat, contained in a small glass-stoppered bottle, add 40 ml. of *glacial acetic acid* and either 2 g. of *anhydrous barium iodide* or 2 ml. of *50% aqueous potassium iodide solution.* Shake for 2 minutes, allow to stand 2 minutes, then shake 2 minutes longer, and pour into 100 ml. of water. Rinse the bottle with 20 ml. of water and titrate with *standard 0.1 N sodium thiosulfate solution*, using *starch solution* as indicator.

Blank. Proceed as above, omitting the addition of the oil.

(*b*) *Rancidity Strong.* To 10 g. of the sample in a strong 60-ml. glass-stoppered bottle, add *glacial acetic acid* and *anhydrous barium iodide* as under (*a*). Displace the air with *carbon dioxide*, insert the stopper, and heat the bottle in boiling water for 2 minutes. Remove from the water, wrap with cloth, shake 2 minutes, then again place in boiling water. After 2 minutes, remove from the boiling water, shake 2 minutes, pour immediately into 150 ml. of cold water, add a few drops of *starch solution*, and titrate with *thiosulfate*, as directed under (*a*).

Blank. Make a blank determination on the reagents and correct accordingly.

Method B (Rancidity Due to Exposure to Air at High Temperatures). To 1.25 to 2.5 g. of the sample contained in a 150-ml. distilling flask, add 10 to 20 g. of *barium iodide* and 100 ml. of *acetic acid*, then displace the air with *carbon dioxide*. Heat to boiling and maintain at gentle boiling for 30 minutes, continuing the flow of *carbon dioxide*. Remove the flame, close the flask tightly to avoid sucking back of the air, immerse in cold water with the inlet tube still open to *carbon dioxide*, and shake gently until cool. Pour into 400 ml. of cold water containing *starch solution* and *dilute sulfuric acid*, then titrate as above.

CALCULATION. Express results as grams of reducible oxygen per 10 or 1000 g. of oil. One liter of 0.1 N thiosulfate solution is equivalent to 1 liter of 0.1 N oxygen solution, and 1 ml. of 0.1 N oxygen solution contains 0.0008 g. of oxygen. If in the titration X ml. of 0.1 N thiosulfate per 10 g. of oil is used, 10 g. of oil will contain $X \times 0.0008$ g. of oxygen. The results can be expressed as (*a*) easily reducible oxygen or (*b*) difficultly reducible oxygen.

EXAMPLES. The following amounts of thiosulfate were required for 10 g. of peanut oil in different stages of rancidity: fresh, 2 to

3 drops; sweet but pink by Kreis test, 1.4 ml.; not sweet, strongly red by Kreis test, 10.5 ml.; rancid, deep red by Kreis test, 66 ml.

Gangl and Rumpel Iodometric Method.[121] REAGENTS. *Starch Solution.* Rub up 2 g. of soluble starch with 10 mg. of $HgCl_2$ and dissolve in boiling water. Reject if the color with iodine is violet (not blue).

Standard Potassium Iodate Solution, 0.01 *N.* Immediately after recrystallizing and drying at 150° to constant weight, weigh out 107.02 g. of KIO_3, dissolve in water, and make up to 1 liter.

PROCESS. Weigh 10 g. of the fat or oil (5 g. if badly rancid) into a 200-ml. glass-stoppered bottle, add 5 ml. of *0.44% potassium iodide solution in propanol,* and 2 drops of *acetic acid.* Heat in a water bath at 50° for 10 minutes, shaking vigorously at intervals, then add 20 ml. of *1 + 1 hydrochloric acid* and shake as before. After allowing to stand for a few minutes, filter on a small moistened paper and wash the flask and filter with several portions of the *acid,* taking care that the fat does not pass through the filter, although a small amount of fat causing a slight turbidity does not seriously becloud the end-point.

Titration. To the filtrate, add 10 ml. of *10% potassium cyanide solution* and 3 ml. of *starch solution,* then titrate with *standard 0.01 N potassium iodate solution.* The disappearance of the color marking the end-point is sharp.

The titration may be carried out in the presence of the fat, but the end-point is not so sharp, especially if the spoilage is marked, because of the slow removal of the iodine absorbed by the fat. After some experience, however, the direct titration is quite satisfactory, the end-point being when the first drop decolorizes the aqueous layer, the subsequent reappearance of the blue color being ignored.

CALCULATION. Use the formula: 1 ml. of 0.01 N iodate solution = 1.66 mg. of potassium iodide. If in the standardization, in which 5 ml. of the propanol solution of potassium iodide are used, B ml. of the iodate solution are used and for the solution derived from 10 g. of the sample F ml. are required in the presence of the fat, then the spoilage number S may be calculated by the formula:

$$S = (B - F) \times 1.66$$

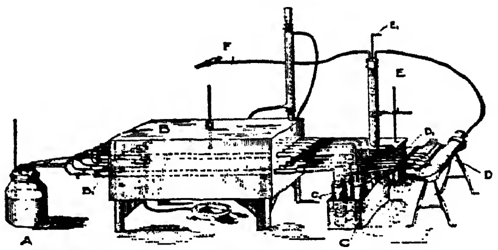
EXAMPLES. Gangl and Rumpel (Vienna) report results on *acid number* and *spoilage number* respectively as follows: lard, normal 0.9 to 1.5 and 3.9 to 4.6, spoiled 1.7 to 48.0 and 9.1 to 18.4, heated to 100° 0.7 and 4.3, overheated 0.7 and 10.6; butter fat, normal 1.3 and 4.8, rancid 4 and 17.7 (also 109.4 and 5.5); cocoanut fat, normal 0.2 and 4.9, rancid 4.3 and 21.2; hardened peanut oil, normal 0.4 and 4.1, rancid 5.2 and 18.6; olive oil, normal 0.9 and 4.3, rancid 8.2 and 16.7; peanut oil normal 0.2 and 3.9, rancid 10.8 and 18.4; soy bean oil, normal 1.1 and 4.3.

OXIDATIVE RANCIDITY

Grettie and Newton Aeration-Permanganate Volumetric Method.[122] The forerunners of this method for measuring susceptibility to rancidity are (1) the Bailey and Ebert method,[123] in which filter paper saturated with the fat is aerated in a tube and the products are collected in decolorized fuchsin (Shiff reagent), and (2) the Issoglio permanganate method,[124] in which the fat is distilled in steam and the distillate, collected in permanganate solution, is titrated with standard oxalic acid solution. The authors (with Swift & Co., Chicago) state that the best-known accelerated rancidity methods are (1) the Greenbank and Holm oxygen absorption method,[125] (2) the Bailey and Ebert method [123] and (3) incubation followed by organoleptic inspection. The Kreis test may be used as an auxiliary indicator.

APPARATUS. *Oxidation Train* (Fig. 112). The parts are: *A*, a wash bottle containing

acid permanganate solution connected with B_1 by individual tubes passing through rubber stoppers; B_1, glass oxidation tubes, containing the fat absorbed in pleated filter paper, extending through metal tubes in a water oven (B); C_1, absorption test tubes in a constant temperature bath (C); D, a manifold device; E, a regulating test tube for maintaining a constant vacuum by raising or lowering tube E_1, and finally the tube F leading to the vacuum source. The oxidation tubes (B_1) have an inside diameter of 20 mm.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1931, 3, 172

FIG. 112. Grettie and Newton Oxidation Train.

and are narrowed at one end to connect with the tubes leading to the absorption test tubes (C_1) which have an inside diameter of 25.4 mm. The water oven is 50 x 35 x 20 cm.

PROCESS. *Aeration.* Remove tubes B_1, soak with *oxidizing mixture,* wash thoroughly, and dry. Crease three times pieces of filter paper 5 x 30 cm. to form 4 layers, weigh 2 g. of the sample on each, and insert in tube. Place in each condensation tube 10 ml. of *0.01 N potassium permanganate solution* to which has been added 1 ml. of *5 N sulfuric acid.* Connect the parts, maintaining the oven at 100°, and start a stream of air through the system at the rate of 1 ml. per second, regulating the flow by a screwcock on tube F. At the end of 20 minutes, replace the condensation tubes by others.

Titration. Add to each tube 11 ml. of *0.01 N oxalic acid solution* and heat on the water bath until completely decolorized, then titrate while still hot with *standard 0.01 N potassium permanganate solution* until a faint pink color appears. Repeat these replacements and titrations at 20-minute intervals until the rate of decomposition of the fat is sufficient to use up 1 ml. of the *permanganate solution* in one 20-minute period.

EXAMPLES. Three fats showed the following number of milliliters of permanganate reduced: 20 minutes, 0.1, 0.8, 0.7; 30 minutes, 0.1, 0.3, 0.5; 60 minutes, 0.3, 0.4, 1.3; 80 minutes, 0.5, 1.0, . . .; 100 minutes, 1.2, 1.9, . . ., respectively. The second sample was a highly flavored fat, the third a very unstable fat.

PEROXIDES

Wheeler Iodometric Peroxide Method.[126] Although the amount of peroxide is not claimed by Wheeler to be an infallible index of keeping quality, it does indicate the extent to which oxidation has progressed. In general a high peroxide value accompanies a high Kreis reaction, although it is not a peroxide that reacts with phloroglucinol to form the red color.

PROCESS. Dissolve 3 to 10 g. of oil in 50 ml. of *6 + 4 acetic acid-chloroform mixture,* add 1 ml. of *saturated potassium iodide solution,* and stir by rotary motion. After 1 minute, add 100 ml. of water and titrate the liberated iodine with *standard 0.1 or 0.01 N sodium thiosulfate solution,* using *starch indicator.* Express results as moles of peroxide per 1 kilo of oil.

The method has been used in the study of auto-oxidation of cottonseed oil and corn oil.

White[127] considers the Kreis test and the peroxide oxygen test as the best means for the estimation of rancidity.

e. Individual Oils

Color Reactions and Characteristics of Individual Oils. The identification of most fats and oils or the judgment as to their

purity depends in most instances on the consideration of evidence obtained in the determination of one or more values; a few oils, however, contain characteristic substances that may be separated and identified or which react when a color test is applied directly to the oil.

Olive Oil

It is fortunate that, although olive oil, the most valuable of food oils, cannot be identified by specific reactions, the presence of the commonest adulterants, notably cottonseed oil and sesame oil, may be detected by reliable color tests; peanut oil may be detected by the separation of arachidic acid.

Cottonseed Oil

The two following methods of testing for cottonseed oil are based on the presence of different minor constituents.

Halphen Sulfur-Amyl Alcohol Test.[128] This test gives a highly characteristic color with normal cottonseed oil, but a less distinct color if the oil has been heated at about 200° and none if heated to 250° or higher. Lard made from the fat of hogs fed on cottonseed products often gives a reaction.

Reagent. *Halphen Reagent.* Dissolve 1 g. of sulfur in 100 ml. of CS_2 and mix with 100 ml. of amyl alcohol.

Process. Add to 3 ml. of the oil or melted fat contained in a test tube an equal volume of *Halphen reagent* and heat in a boiling saturated brine bath. If normal cottonseed oil is present, even in as small an amount as 1%, a red color appears in 15 minutes, although longer heating may intensify the color.

For the same lot of oil and same time and temperature of heating, the color is approximately proportional to the content of cottonseed oil in the mixture. The color persists for days; this is not true of other color tests for oils.

Gastaldi Pyridine Modification.[129] The constituent of amyl alcohol that reacts with cottonseed oil in the Halphen test was found by Gastaldi to be pyridine. He conducts the test as follows.

Heat in a water bath for 15 minutes 5 ml. of the oil with 4 ml. of *carbon bisulfide*, containing 1% of *sulfur*, and 1 drop of *pyridine*. The color that forms if the oil contains cottonseed oil is the same as that produced in the Halphen test.

Bechi-Dudley Silver Nitrate Test. Of various modifications, that proposed by Dudley as described by Wesson[130] is here given. The test is not reliable in all cases, but is useful as corroborating the Halphen test or as furnishing tentative evidence where the latter, owing to heating of the oils, fails. Wesson recommends that in the examination of steam lard, which gives a slight reaction, 50 g. of the sample be purified by shaking with 25 ml. of *2% nitric acid* and washing and that the disturbing influence of acidity be overcome by shaking with *dilute sodium hydroxide solution*, washing with water, and finally with *2% nitric acid*.

Reagent. Dissolve 2 g. of $AgNO_3$ in a mixture of 200 ml. of ethanol and 40 ml. of ether. Expose to sunlight until reaction ceases, then decant into a dark bottle.

Process. Heat on a boiling water bath for 15 minutes with stirring 10 ml. of the oil or melted fat with 5 ml. of the *reagent*. In the presence of cottonseed oil, the oil darkens and a silver mirror is formed.

Gossypol

(In Cottonseed Products)

Carruth Dianiline Gravimetric Method.[131] Several modifications have been developed at the North Carolina Experiment Station.

I. Smith and Halverson Modification.[132] **A. Total Gossypol.** Process. *Naphtha Extraction.* Weigh 2 g. of the sample, ground to pass a 40-mesh sieve, into a 25-ml. medium

or porous alundum filter crucible and extract in the Pickel apparatus for 3 hours with *naphtha* to remove the crude oil. Dry the crucible and meal, then stir and place the crucible and contents in a clean Pickel flask.

Aniline Precipitation. Add to the meal from a pipet by suction 5 ml. of *aniline*, place 15 ml. of *90% ethanol* in the flask, and extract for 24 hours in the Pickel extractor. Transfer the extract to a 200-ml. Erlenmeyer flask, washing with *ether* through a funnel. The raffinose, which is precipitated by the ether, goes into solution during the precipitation. Remove any crystals of gossypol adhering to the Pickel flask to a weighed Gooch crucible by means of water.

Ethylene Glycol Digestion. Remove the ethanol and ether under reduced pressure, then add to the residue 7 ml. of *ethylene glycol* and 10 ml. of *ether* and digest on the steam bath. When the ether begins to boil, add water with shaking at 3-minute intervals in the following amounts: 0.5, 0.5, 1.5, and 2.5 ml. Digest gently 15 to 20 minutes and allow to stand overnight. The precipitate of dianiline gossypol thus formed is coarse and readily handled.

Filter on the Gooch crucible previously used, wash with *50% ethanol*, then with a small amount of *naphtha*, followed by 5 ml. of *95% ethanol*, and finally four times with water. Dry at 100° to constant weight.

CALCULATION. Multiply the weight of dianiline gossypol by the factor 0.775, thus obtaining the weight of total gossypol.

B. Bound and Free Gossypol. The procedure has been further modified as follows.

II. Smith Modification for Bound and Free Gossypol.[133] By the use of peroxide-free ether containing ethanol and water, the full amount of free gossypol is extracted.

REAGENT. *Ether-Ethanol Mixture.* Adjust peroxide-free absolute ether so as to contain 2.3 to 2.5% of ethanol and 1 to 1.2% of water.

PROCESS. *Sample.* Stir 120 g. of the sample, ground to pass a 40-mesh sieve, with 27.5 ml. of water in a mortar. Incorporate the pasty part with the dry, pass through a 20-mesh sieve, and use this portion in working up the wet meal until all passes through the sieve.

Determine moisture, which should be about 22%. Divide the remainder of the sample into two equal parts.

Ether Extraction. Proceed with each part as follows. Extract for 72 hours in a Soxhlet extractor with *ether-ethanol mixture*. Add 5 ml. of water to the extraction flask.

Filtration. Collect the ether in the extraction chamber of the Soxhlet apparatus, leaving only 25 to 30 ml. of liquid in the flask. Filter the extract through a Gooch crucible fitted with *Hy-Flo-Supercel* (Johns-Manville Co.) over ignited asbestos, collecting the filtrate in a 200-ml. Erlenmeyer flask under a bell glass. Wash with *ether* and combine washings with the filtrate.

Naphtha Precipitation. Remove the ether from the filtrate by heating in a pan of water at 50° and applying suction, add 75 ml. of *naphtha* and 5 ml. of *ethylene glycol*, mix, and allow to stand overnight. Filter into an Erlenmeyer flask through a Gooch crucible, prepared as above to remove the naphtha precipitate, and wash with a small amount of *naphtha*, keeping the filtrate in a pan of water at 35° to prevent further precipitation.

Dianiline Precipitation. Add to the filtrate 2 ml. of *aniline*, shake well, digest in water at 50 to 55° for 60 to 75 minutes, and add *naphtha* to make the total volume at 50 to 60 ml. Stopper loosely, allow to stand overnight, or at least 36 hours if the precipitate is small (10 to 15 mg.). Collect the dianiline gossypol on a tared Gooch crucible, wash with *naphtha*, followed by four washings with water, and 5 ml. of *ethanol*. Dry the precipitate to constant weight at 100°.

CALCULATION. Multiply the weight of dianiline gossypol by 0.775 to obtain the corresponding weight of soluble gossypol.

III. Halverson and Smith Revised Modification for Oil.[134] APPARATUS. *Shaking Constant Temperature Water Bath.*

REAGENTS. *High Gossypol Oil.* Extract prime cottonseed meats with peroxide-free ether. Remove the greater part of the ether by distillation in hot water kept below 60°, then, to remove the last traces of ether, filter the hot oil through glass wool and gently heat under reduced pressure. Store in the refrigerator at 5°.

Pyridine, b.p. 115 to 116.3°. Fraction Eastman "practical 112 to 117°" to eliminate water.

Naphtha-Pyridine Wash Liquid. Mix 100 parts of naphtha with 3 parts of dry pyridine.

Wesson Refined Cottonseed Oil.

PROCESS. *Clarification.* Filter the sample by gravity through glass wool and weigh 25 g. into a 250-ml. Erlenmeyer flask. Into the same flask pipet 5 ml. of *high gossypol oil,* wiping off all the oil from the outside of the pipet before adjusting to the mark. After delivering the oil, rinse the inside of the pipet with *naphtha* (b.p. 30 to 60°). Into another Erlenmeyer flask, containing 25 g. of *Wesson refined cottonseed oil,* pipet 5 ml. of the *high gossypol oil.* To each charge, add 75 ml. of *naphtha* and, after allowing to stand overnight in lightly stoppered flasks, add 0.1 g. of *Hy-Flo-Supercel.* Filter with suction through dry Gooch crucibles packed with *Hy-Flo-Supercel* over asbestos into 250-ml. Erlenmeyer flasks, placed under bell jars, and wash with 10 to 15 ml. of *naphtha.*

Aniline-Pyridine Treatment. Add to each flask 4 ml. of *aniline* and 12 ml. of *fractionated pyridine,* attach Hopkins condensers, and agitate 72 hours in a shaking constant temperature water bath held at 43 to 46°. Attach to the lower end of each condenser stopper a small cup to catch any drops of water which form in the condenser tube, thus preventing adhesion of the precipitate to the glass. After the shaking, allow to rest 1 hour at room temperature, decant off a portion of the solution for use in washing out adhering precipitate, then collect the precipitate in Gooch crucibles and wash with the *100 + 3 naphtha-pyridine wash liquid.* Finally wash down the sides of the crucibles, fill the crucibles half full with the wash liquid five times, again wash down the sides, and wipe the outside with a cloth. Dry the precipitate in an oven at 110° for 18 to 24 hours.

CALCULATION. Subtract from the total weight of dianiline gossypol in the charge that added in 5 ml. of high gossypol oil, multiply the remainder by 0.775, thus obtaining the equivalent amount of gossypol, which, multiplied by 4, gives the percentage in the crude oil.

Royce Pyridine-Aniline Gravimetric Method.[135] The following modification which takes advantage of the changes introduced by Royce and Kibler[136] and the criticism of Smith and Halverson,[137] is proposed.

Royce, Harrison, and Deans Modification.[138] PROCESS. *Precipitation of Gossypol.* Weigh 50 g. of filtered crude cotton-seed oil into a 200-ml. wide-mouth extraction flask and dilute to 110 to 130 ml. with *naphtha.* Add 10 ml. of *4 + 1 pyridine-aniline reagent* and mix well by swirling. Stopper loosely and store in a warm place (35 to 40°) for several hours, replacing any *naphtha* lost by evaporation, then stopper and hold at room temperature for 7 to 14 days, the longer period being for a gossypol content below 0.1%.

Filtration. Collect the precipitate of dianilinogossypol-dipyridine in a Gooch crucible, rinse, and wash with *naphtha containing 1 to 3% pyridine,* keeping the volume of the filtrate below 100 ml.

Drying and Weighing. Heat the crucible and precipitate for 1 hour at 160° to remove the pyridine of crystallization and weigh as dianilinogossypol, or dry for 2 hours at 60° and weigh as dianilinogossypol-dipyridine.

CALCULATION. Convert the weight of ...ogossypol or dianilinogossypol-di-

pyridine to gossypol by the factors 0.775 or 0.627 respectively.

NOTE. Podol'skaya[139] determines the gossypol in cottonseed meal and cake by extraction of 30 g. of the sample for 5 hours with *peroxide-free ether*, evaporation of the ether, and treatment of the extract with 10 ml. of *naphtha* (b.p. 60 to 100°), 2 ml. of *pyridine*, and 1.5 ml. of *aniline*. If the gossypol content of the sample is over 0.1% the precipitation is complete in 30 to 40 minutes, if between 0.08 to 0.1% in 2 to 2.5 hours, and if 0.05 to 0.06% in 24 hours. The precipitate is dried for 1 hour at 100°.

Lyman, Holland, and Hale Butanol-Aniline Colorimetric Method.[140] The method was developed at the Texas Agricultural Experiment Station, College Station.

APPARATUS. *Butt Extractor*, with 250-ml. Erlenmeyer flask.

Cenco Sheard Spectrophotelometer, or any photoelectric colorimeter.

PROCESS. *Ether Extraction.* Wrap 2 g. of the sample, ground to pass a 40-mesh sieve, in a 125-mm. S. & S. No. 597 filter paper and rewrap in a second paper, the top of which is left open except for a plug of absorbent cotton to distribute the solvent. Extract in a Butt extractor with 60 ml. of *peroxide-free ether*, containing 2.3 to 2.5% of *ethanol* and 1 to 2% (by weight) of water, for 72 hours at the rate of at least 150 drops per minute, into a 250-ml. Erlenmeyer flask.

Color Formation. Add 5 ml. of *n-butanol* to the ether extract and remove the ether by heating in a pan of warm water under reduced pressure. Transfer the residue to a 25-ml. volumetric flask by means of *n-butanol* and fill to the mark with *n-butanol*. Transfer 2 aliquots of 2 to 5 ml., depending on the gossypol content, to two other 25-ml. volumetric flasks, then add to one 2 ml. of freshly distilled *aniline* and dilute both to the mark with *n-butanol*.

Color Reading. After about 20 minutes, transfer the two solutions to the cells of the instrument and make relative measurements of the light transmission at 440 mμ wave length.

CALCULATION. The amount of gossypol is directly proportional to log (I_0/I_1) in which I_0 and I_1 are the intensity of light transmission without and with aniline respectively. Compare with a standard curve prepared by the use of values obtained with pure gossypol isolated by the Campbell, Morris, and Adams process.[141]

OIL

The identification of peanut oil and its detection in other oils depends chiefly on the isolation of a mixture of arachidic and lignoceric acids, its characteristic constituents, a process based on lead soap precipitation as first carried out by Rénard.[142] Several modifications of the Rénard method have been described by European and American chemists and have been subjects for extended controversies. The most important modifications are those of Tortelli and Ruggeri,[143] Archbutt,[144] Evers,[145] and Tolman.[146]

Rénard-Tolman Lead Soap Gravimetric Method. By the Tolman modification, 5 to 10% of peanut oil in other oils may be detected. Jamieson[147] warns that a small amount of arachidic and lignoceric acids may be due to rape oil which sometimes contains over 2% of lignoceric acid. Olive oil contains traces of arachidic acid.

PROCESS. *Saponification.* Heat 20 g. of the sample in an Erlenmeyer flask under a reflux condenser with 200 ml. of *ethanolic potassium hydroxide solution* for 30 minutes as in the determination of saponification number.

Lead Soap Precipitation. Cool, neutralize exactly with *25% acetic acid*, using *phenolphthalein indicator*, and pour into a boiling mixture of 100 ml. of water and 120 ml. of *20% normal lead acetate solution.* Boil for 1 minute, then cool the flask in water, rotating to distribute the soap over the interior sur-

face. Decant off the aqueous liquid and wash with cold water, and then with *90% ethanol.*

Ether-Insoluble Lead Soap Crystallization. Add to the soap 200 ml. of *ether*, cork, allow to stand until the soap disintegrates, and boil for 5 minutes on a water bath under a reflux condenser. The soap from most oils will dissolve, that from lard, however, because of the high stearin content, will remain out of solution. In either case, cool to 15 to 17° and allow to stand about 12 hours until the insoluble soap separates out. Collect the crystallized lead soap on a filter, wash thoroughly with *ether*, following the technique given under Saturated and Unsaturated Acids, reserving the filtrate if desired for determination of the soluble fatty acids.

Fatty Acids of Insoluble Lead Soap Separation. Wash the lead soap back into the flask with a stream of hot water acidified with *hydrochloric acid*, add an excess of *10% hydrochloric acid*, partially fill the flask with hot water, heat, and allow the fatty acids to collect on the surface. Fill the flask with hot water and allow the fatty acids to harden and the lead chloride to deposit. Pour off the lead chloride and acid solution, add a fresh portion of hot water, cool, and pour off the liquid, repeating the operation until the acid is removed.

Fatty Acids of Insoluble Lead Soap Crystallization. Dissolve the fatty acids in 100 ml. of boiling *90% ethanol*, cool to 15° with agitation to aid crystallization. Collect the crystals on a filter, wash twice with 10 ml. of *90% ethanol*, and then with *70% ethanol*. Dissolve in and wash with boiling *absolute ethanol*, collecting the solution in a tared dish, evaporate the ethanol, dry at 110°, cool, and weigh.

CALCULATION. Add to the weight 0.0025 g. for each 10 ml. of 90% ethanol used in crystallization and washing if at 15° and 0.0045 g. if at 20°. The melting point of the arachidic acid thus obtained should be 71 to 72°. Multiply the weight by 20 to calculate the approximate amount of peanut oil.

Observe the form of the arachidic acid crystals under the microscope.

NOTE. The directions given above apply to the present (1940) official A.O.A.C. procedure, except that the following should be substituted for the paragraph with the same side head:

Fatty Acids of Insoluble Lead Soap Separation. Collect the insoluble lead soap on paper fitted to a Büchner funnel, wash thoroughly with *ether*, then transfer the soap with a jet of *ether* into a separatory funnel, removing any of the soap that adheres to the paper with *10% hydrochloric acid.* Make up to about 200 ml. with the *acid* and add *ether* to make its total 150 to 200 ml. Shake vigorously for several minutes, allow to separate, and run off the acid layer. Wash the ether solution once with 100 ml. of the *acid*, then with water until the washings are neutral to *methyl orange.* Break up any decomposed lumps of lead soap after the third washing by shaking with a little of the *acid*, then wash until the washings are neutral. Remove the ether by distillation and dry after adding a little *absolute ethanol* and evaporating on the steam bath.

Bellier Acid-Ethanol Test.[148] This test is not official in the United States.

REAGENTS. *Potassium Hydroxide Solution*, ethanolic. Dissolve 85 g. of KOH in *70%* ethanol and make up to 1 liter.

Acetic Acid. Dilute 29 g. of glacial acetic acid to 100 ml. and adjust so that 1.5 ml. neutralize 5 ml. of the alkali solution.

Acid-Ethanol. Acidify with HCl and dilute ethanol so that the mixture will contain 1% of the acid and 70% of the ethanol.

PROCESS. *Saponification.* Boil gently for about 2 minutes over a free flame 1 g. of the sample in a test tube with 5 ml. of *ethanolic potassium hydroxide solution* until saponification is complete.

Neutralization. To the clear saponified so-

lution, add 1.5 ml. of *acetic acid*, shake, and cool in water at about 18° not less than 30 minutes with shaking.

Ethanol Precipitation. Add 50 ml. of *acid-ethanol*, shake, and again cool in water for 1 hour.

Overlook any opalescence and observe whether a flocculent precipitate, indicative of peanut oil, appears. Olive oil from certain regions may show a slight precipitate, but this disappears on reheating and cooling.

NOTE. Lepierre and De Carvalho [149] report that the 1930–31 oils, produced in certain Spanish and adjoining Portuguese districts, gave Bellier reactions due to an excess of tripalmitin and tristearin together with traces of arachidin and lignocerin which falsely indicated the presence of 5 to 10% of peanut oil.

Bellier Quantitative Method.[150] REAGENTS. As in the Bellier test.

PROCESS. Saponify as above, but use 5 g. of the oil and 25 ml. of the *alkali solution*, in a 250-ml. Erlenmeyer flask. Exactly neutralize with *acetic acid*, cool in water, allow to stand 1 hour, and collect the precipitate on a 9-cm. filter, then wash with *acid-ethanol* at about 18°. Dissolve the precipitate from the filter with about 30 to 50 ml. of boiling *ethanol*, reduce the ethanol strength to about 70% by adding water, allow to stand 1 hour at 18°, filter, wash with *acid-free 70% ethanol*, dry at 100°, and weigh.

Evers Modification.[151] REAGENTS. *Potassium Hydroxide Solution*, ethanolic. Dissolve 80 g. of KOH in 80 ml. of water and make up to 1 liter with 90% ethanol.

Acid-Ethanol. Prepare as for the Bellier Test above.

PROCESS. *Saponification.* Saponify 5 g. of the sample with 25 ml. of *ethanolic potassium hydroxide solution* by heating for 5 minutes under a reflux condenser.

Neutralization and Precipitation. While hot add 7.5 ml. of *1 + 2 acetic acid* and 100 ml. of *acid ethanol*, allow to stand for 1 hour at 12 to 14°, filter, and wash with *acid ethanol*, keeping at 17 to 19° until the filtrate does not become turbid with water and noting the amount used.

Crystallization. Dissolve the precipitate in 25 to 70 ml. of hot *90% ethanol* and allow to crystallize at a definite temperature between 15 and 20° for 1 to 3 hours. Collect the crystals on a filter, wash with a definite volume of *90% ethanol*, using about half the amount required for solution previous to crystallization, then with 50 ml. of *70% ethanol*, wash with warm *ether* into a weighed flask, distil off the ether, dry in a boiling water oven, and weigh. Recrystallize from *90% ethanol* if the melting point is lower than 71°.

CORRECTION. Correct for solubility in 90% ethanol as in the Rénard Test above and for solubility in the total amount of 70% ethanol used in all steps of the process as follows.

Found (Grams after Correcting for Solubility in 90% Ethanol)	Dissolved in 100 Ml. of 70% Ethanol		
	Melting Point		
	71°	72°	73°
Less than 0.02	0.006	0.005	0.004
0.02 to 0.05	0.007	0.006	0.005
0.05 to 0.08	0.009	0.007	0.005
0.08 to 0.10	0.011	0.007	0.006
More than 0.10	0.013	0.008	0.006
Factor for peanut oil	17	20	22

In case little or no arachidic acid and lignoceric acid crystallize out from the 90% ethanol, add water to 70% ethanol content, allow to crystallize at 17 to 19° for 1 hour, filter, wash with *70% ethanol*, and proceed as before, correcting for solubility in the 70% ethanol. If the melting point is below 71°, add a small amount of *90 or 70% ethanol* and recrystallize.

Sesame Oil

A substance is present in sesame oil that, on addition of furfural and hydrochloric acid, forms a crimson coloration. In the Baudouin test the furfural is formed by the action of the acid on sugar; later Villavecchia explained the reaction and substituted furfural for sugar.

Baudouin Sugar-Acid Test.[152] Dissolve 0.1 to 0.2 g. of *powdered sugar* in 10 ml. of *hydrochloric acid*, add 10 ml. of the oil, shake violently for 1 minute, and allow to stand. If sesame oil is present, even in small amount, a crimson color appears in the acid layer.

Certain lots of sesame oil from the Mediterranean region, believed to be genuine, give a light pink color, but it is claimed that if the test is applied to the fatty acids such oils give a negative test.

Villavecchia and Fabris Furfural-Acid Test.[153] Shake thoroughly 10 ml. of the oil with 10 ml. of *10% hydrochloric acid* and 0.1 ml. of *2% furfural in ethanol*. If sesame oil is present, the same color develops as in the Baudouin test.

In the methods of the A.O.A.C. it is stated that if the color formed in 10 minutes disappears on shaking with 10 ml. of water, sesame oil is not present.

Notes. The German Official Test,[154] as adopted in 1908 for butter, consists in shaking vigorously for 30 seconds 5 ml. of the melted fat, 5 ml. of *naphtha*, and 0.1 ml. of a *1% solution of furfural in absolute ethanol*.

Weigmann,[155] Soltsien,[156] and Lauffs and Huismann[157] found that the presence of rancid fat prevents or interferes with the color reaction. The last-named authors state that addition of cottonseed oil counteracts the disturbing influence of rancidity.

Zimmermann[158] notes that over-refining may interfere with the furfural test, but to a lesser extent with the Soltsien test.

Bishop[159] observed that sesame oil exposed for some time to air gives various shades of green and blue with acid, hence the color obtained by the furfural reaction will be modified to some extent. Kreis[160] employs this reaction as a means of detecting rancidity in fats, using in his test fresh sesame oil.

Soltsien Test. The advantage of this test is that the azo colors that become red with acid do not interfere. It is carried out according to the German oleomargarine regulations as follows.[154]

Reagent. *Stannous Chloride Solution*, fuming. Saturate a mixture of 5 parts of crystallized $SnCl_2$ and 1 part (by weight) of HCl gas with dry HCl and filter through asbestos. After settling, keep in small glass-stoppered bottles filled to exclude air.

Process. Dissolve 5 ml. of the melted fat in 10 ml. of *naphtha*, add 2.5 ml. of strongly fuming *stannous chloride solution* (Bettendorff reagent), shake until mixed, but not longer, place in a water bath at 40° until the liquids separate, then immerse in water at 80° up to the line of separation.

If sesame oil is present, the stannous chloride solution becomes red in 3 minutes.

Pavolini Acid-Ethanol Test.[161] Reagent. Mix 80 g. of 98% sulfuric acid, 10 g. of ethanol, and 10 g. of water.

Process. Dissolve 5 ml. of the sample in 5 ml. of *ether*, add 2 ml. of the *reagent*, shake, and let stand. If sesame oil is present, the lower layer acquires the distinct green color and fluorescence of sesame oil. With 5%, the color is light green; even with 1%, the green color is evident.

Cruciferous Oils

Erucic acid is characteristic of oils of cruciferous seeds, such as rape, mustard, and charlock, and tests based on its presence are the only certain means of detection of these oils in olive and other more valuable oils, although low saponification and high iodine numbers add confirmation.

The following methods have been found useful. Others, also depending on the sepa-

ration of the solid and liquid acids as lead soaps, have been devised by Holde and Marcusson,[162] and by Kreis and Roth.[163]

Tortelli and Fortini Method.[164] The test was devised at a Genoa laboratory.

PROCESS. *Saponification.* Reflux 20 g. of the sample with 50 ml. of *12% ethanolic potassium hydroxide* and neutralize to *phenolphthalein* with *10% acetic acid.*

Lead Precipitation. Pour slowly into 300 ml. of boiling *7% normal lead acetate solution,* cool in water, rotating the flask to cause the soap to cling to the sides of the flask, decant the liquid, and wash with three 200-ml. portions of water at 60 to 70°. Drain and dry the residue with a clean cotton cloth.

Ether Extraction. Warm with 80 ml. of *ether* for 20 to 30 minutes under a reflux condenser with repeated shaking. Stopper the flask and cool for exactly 1 hour at exactly 15°. Decant carefully the clear ether solution through a filter into a separatory funnel, avoiding evaporation. Add to the residue in the flask 40 ml. of *ether,* heat, cool, and decant as before, then wash the residue onto the filter with another portion of 40 ml. of *ether.* Transfer the insoluble lead soaps to another separatory funnel with 100 ml. of *ether.*

Fatty Acids Liberation from Lead Soap. Shake the contents of both separatory funnels with 150 ml. of *20% hydrochloric acid,* allow to separate, and draw off the acid together with the precipitated lead chloride. Treat once or twice more with *acid,* using 100 ml., and wash twice with 100 to 150 ml. of water, avoiding an emulsion. Filter the ethereal solutions of both the liquid fatty acids (from the ether-soluble lead soap) and the solid (from the ether-insoluble lead soap) into dishes and evaporate the ether at a low temperature.

Determine the melting point and iodine number of the solid and liquid fatty acids.

Sodium Soap Preparation. Dissolve the liquid fatty acids in 40 ml. of *absolute ethanol,* add *saturated sodium carbonate solution* until the salt begins to precipitate, evaporate nearly to dryness, and complete the drying in a vacuum desiccator over sulfuric acid.

Warm the residue in 40 to 50 ml. of *absolute ethanol,* filter through a hot funnel, and repeat the treatment three or four times. Cool the combined ethanolic filtrates, collect the soap which separates on a paper, suck dry by means of a pump, and finish the drying (which must be thorough) over sulfuric acid.

Turbidity Test. Place 0.5 g. of the thoroughly dry sodium soap, together with 20 ml. of *absolute ethanol,* in a large test tube, suspend the tube by means of a perforated stopper in an Erlenmeyer flask filled with water, introduce a thermometer into the test tube, heat until the soap dissolves, using the thermometer as a stirrer, then cool until crystals form throughout the liquid, noting the critical solution temperature (C.S.T.) at that moment.

Determine also the critical temperature of 5 ml. of the total fatty acids in 10 ml. of *70% ethanol.*

EXAMPLES. The following results by Tortelli and Fortini illustrate the value of the process.

	Fatty Acids			
	Solid		Liquid	Total
	m.p.	Iodine No.	Sodium soap	Fatty acids in 70% ethanol
	°C.		C.S.T.	C.S.T.
Olive	58–59	7.8	20–24	16–36
Rape	41–42	62.0	45–50	80
Olive 50%, rape 50%	47–48	32.0	35–40	..
" 70 " 30	48–49	28.0	30–35	..
" 80 " 20	50–51	22.1	30–35	..
" 90 " 10	54–55	12.8	30–34	..
Sesame	55–56	9.2	18–20	53
Peanut	57–58	13.0	18–22	59
Cottonseed	57–58	19.0	14–16	67

Biazzo and Vigdorcik Palladium Hydrogenation Method.[165] The authors (Naples University) endorse the Tortelli and Fortini method, but note that it is laborious. They criticize the method of Holde and Marcusson and of Kreis and Roth. Their own method depends on the conversion by catalysis of erucic into behenic acid, which has a high melting point (84°). Arachidic and lignoceric acids, if present, may be determined in the same portion.

REAGENTS. *Potassium Hydroxide Solution*, 20% in ethanol. Dilute 40 ml. of a 50% aqueous solution with 100 ml. of ethanol, allowed to settle for a day and filtered through glass wool.

Acid Ethanol, 90% containing 10 drops of 1.0 *N* HCl per liter.

PROCESS. *Saponification*. Reflux 20 g. of the sample with 40 ml. of *ethanolic potassium hydroxide solution* and transfer the soap solution with 150 ml. of water to a 500-ml. separatory funnel.

Extraction. Liberate the fatty acids with *dilute sulfuric acid*, extract with 200 ml. of *ether*, draw off the aqueous liquid, and wash the ether solution twice with 150 ml. of water. Dry the ether solution with *calcium chloride* or *anhydrous sodium sulfate*, distil off the ether, and heat for 15 minutes in a water oven in an air current. Warm the fatty acids on the water bath to the boiling point with 180 ml. of *anhydrous acetone*, add 20 ml. of *1.0 N potassium hydroxide solution*, and cool to 15°. Filter by suction, wash carefully with 4 portions of 10 ml. of cold *acetone*, dissolve the residue in water, transfer to a separatory funnel, liberate the fatty acids with *hydrochloric acid*, extract with 100 ml. of *ether*, and draw off the aqueous liquid.

Lead Soap Precipitation. Wash the ether solution twice with 100-ml. portions of water, then shake for 5 minutes with 15 ml. of *30% normal lead acetate solution* and draw off the aqueous solution. Immerse the separatory funnel for 30 minutes in water at 23 to 25° and separate the precipitate of lead soap from the ethereal solution by filtering.

If the amount of insoluble lead soaps warrants, test for arachidic acid as described below.

Deleading. Remove the lead from the ether filtrate by *hydrochloric acid* and wash with water until the acid is removed.

Hydrogenation. Treat with *bolted charcoal containing 2% of palladium*[166] until no more hydrogen is absorbed.

Test for Behenic Acid. Evaporate the ether and subject to fractional crystallization from 50-, 25-, 12.5-, and 5-ml. portions of *acid ethanol*, dissolving by gentle heating and allowing to stand each time for 30 minutes at 15°.

A melting point above 71° indicates, and between 76 and 79° proves, the presence of a cruciferous oil.

Arachidic and Lignoceric Acids Test. Wash the soaps thoroughly with cold *ether*, transfer to a separatory funnel with 200 ml. of *ether*, shake twice with 100 ml. of *20% hydrochloric acid*, wash the ether solution with water until free from acid, dry with *calcium chloride* or *anhydrous sodium sulfate*, filter with additional *ether*, distil the ether from the filtrate, and remove the last traces by a current of air.

Crystallization. Dissolve the fatty acids in 50 ml. of *90% ethanol* containing 10 drops of *1.0 N hydrochloric acid* per liter. Cool for 30 minutes in a bath at 15° and collect the crystals.

Recrystallization. Dissolve in 25 ml. of *ethanol* and proceed as before.

If the separated crystals are arachidic and lignoceric acids, they should have a melting point of 73.5 to 74° as obtained by this method.

Cocoanut, Palm, and Palm Kernel Oils

See Part II, G7 and 13.

Whale Oil

The marine oils include those of mammals, especially species of whale, and of true fishes. Cod liver oil has long been used as a medicinal food because of its high cholesterol content, but other marine oils until recently have been legitimately used only in the arts.

Whale oil, formerly an unappetizing product, since the introduction of hydrogenation, by which process it is converted into a hard fat permitting of refining and deodorizing, has been exploited for the manufacture of butter and lard substitutes. Although it contains unsaturated acids of the clupanodonic series, its iodine number is only slightly lower than that of cottonseed oil owing to the presence of saturated acids.

Detection. The natural oil yields approximately 25% of ether-insoluble brominated glycerides, but in the event that the amount is reduced by hydrogenation, chief dependence must be placed on the test for nickel described above.

Grimme Iodine Test.[167] This test may also be used, although the intensity of the color is inversely proportional to the hardness.

Process. Dissolve 5 parts of the sample in 95 parts of *1 + 1 naphtha-xylene mixture* and shake 5 ml. of the mixture with 1 ml. of *sulfuric acid* containing 1 drop of *iodine tincture*, noting the color after 5 minutes and 60 minutes. A violet-red color indicates whale oil.

Fish Oils

Tests for Fish Oils. Tsujimoto[168] detects the presence of fish oils by the turbidity formed on dissolving 0.5 g. of the separated fatty acids in 10 ml. of *ether*, heating while cooling with 3 to 5 ml. (depending on the iodine number) of *iodomonochloride in acetic acid*, and allowing to stand for 2 hours at 15 to 20° with shaking.

Davidsohn[169] found that the test is applicable only when the limited number of oils tested by Tsujimoto are involved. Cod liver, spermaceti, herring oil, and blown fish oil give negative results, hence the octabromide test of Marcusson and Huber[170] remains the only reliable test for fish oils.

The earlier hexabromide tests appear to have been discredited.

Paraffin

Formerly paraffin was used to some extent as a constituent of butter and lard substitutes, but authentic instances in recent years have not come to light.

Detection. If present in considerable amount, paraffin is evident on saponification, as in the determination in the Köttstorfer method, as a persistent oily layer on the surface of the liquid. A low saponification number and a high percentage of unsaponifiable matter furnish further evidence.

Thompson and Hurst Turbidity Test.[171] Heat 3 ml. of the melted sample with 10 ml. of a *mixture of absolute ethanol and chloroform* (1 + 1) until dissolved, then cool in water. The presence of 1.5% of paraffin will cause a turbidity on standing 3 minutes.

Holde Test. Saponify 10 drops of the sample with 5 ml. of *0.5 N ethanolic potassium hydroxide solution*, add to the hot soap solution 5 ml. of water in 1-ml. portions, noting the appearance after each addition. In the presence of as little as 0.3% of paraffin, the liquid has a silky appearance.

Dunlop[172] states that the test also serves to detect fish oil stearin in tallow, which he finds a common adulterant.

Polenske Test.[173] This test is designed to detect the presence of minute quantities of paraffin added to obscure the results of the phytosterol acetate test for vegetable oils.

Polenske added 0.002 to 0.1 g. of paraffin to 0.1-g. portions of a mixture of phytosterol and cholesterol (94 + 6) and carried out the phytosterol acetate test with the following results.

Added Paraffin	Melting Point		
	3d crop	4th crop	5th crop
None	118.0	119.0	120.0
0.002 grams	117.5	118.5	120.0
0.003 "	117.5	118.0	119.5
0.005 "	116.5	117.0	118.0
0.007 "	115	112	115
0.01 "	113	108	112
0.02 "	104	89	..
0.05 "	79	60	55
0.1 "	64	55	53

From these figures, it is evident that whereas 0.002 to 0.005 g. of the paraffin was without appreciable influence on the detection of phytosterol and 0.02 to 0.1 g. gave abnormally low melting points unmistakably pointing to paraffin, intermediate amounts (0.007 to 0.01) gave melting points like those of pure cholesterol.

To eliminate the paraffin, treat the unsaponifiable matter from 100 g. with 1 ml. of *naphtha* (b.p. below 50°) at 15 to 16° for 20 minutes, filter through cotton wool, wash with five 0.5-ml. portions of *naphtha*, and apply the phytosterol acetate test to the undissolved residue.

To determine the paraffin, prepare the unsaponifiable matter from 100 g. of the fat (or evaporate the naphtha extract obtained above) and destroy sterols by heating once (twice if only a trace of paraffin) with 5 ml. of *sulfuric acid* in a glycerol bath for 1 hour at 104 to 105°. Extract the paraffin with 10-ml. portions of *naphtha*, wash the solution with 3 portions of 10 ml. of water, using a few drops of *barium chloride solution* in the second portion, evaporate, and weigh.

Lewkowitsch [174] notes that if paraffin constitutes as much as 10% of the crude sterols, it will separate as an oily drop on conducting the phytosterol acetate test directly on the original unsaponifiable matter. If the amount of paraffin is not sufficient to form a drop, its presence may be indicated by the melting points as compared with those in the above table: in that event combine all crystals and mother liquors, evaporate to dryness, saponify, and extract with *ether* to recover the original unsaponifiable matter, determining incidentally the saponification number (cholesterol acetate is 135.5), then determine paraffin as directed by Polenske.

3. SALAD DRESSING

Formerly prepared only in the family, hotel, or restaurant kitchen, salad dressing is now a rival of tomato catsup and prepared mustard as a commercial semi-solid condiment.

Composition. The product marketed in glass jars varies greatly in the proportion of ingredients, as evidenced by analyses reported by E. M. Bailey [175] which show 2.02 to 15.5% of egg yolk, calculated from the lipoid phosphorus content, and 56.5 to 87.3% of fat determined by the Röse-Gottlieb-Patrick method (Part II, G1).

A normal mayonnaise (No. 1) and a commercial type of dressing (No. 2), submitted for analysis to members of the A.O.A.C. by Ryan,[176] contained, as calculated from the analysis of the ingredients, the following.

	No. 1	No. 2
Egg yolk	3.62	4.58
Egg white	7.12	7.86
Total egg	10.74	12.44
White in egg component	66.27	63.17
Vegetable oil	54.59	74.21
Vinegar (5% acid)	21.00	10.62
Minor constituents	8.44	3.51
Added water	5.22	−0.79

Analytical Methods. As calculated from the analyses of the dressings, the percentages of the ingredients reported agreed with rea-

sonable closeness to the theoretical composition. The methods were those proposed by Lepper and Vorhes,[177] essentially as given in the following sections and adopted as official by the Association.

Solids

See Part II, H7.

Protein

Heat 15 g. of the sample in a Kjeldahl flask on the steam bath until the egg is thoroughly cooked and the oil separates readily. Cool, shake with 50 ml. of *naphtha*, and decant the naphtha solution through a small filter, repeating twice. Wash the filter paper with *naphtha* and add to the sample. Add 35 ml. of *sulfuric acid* and determine nitrogen by the Kjeldahl Method (Part I, C1*c*).

Oil

Acid Hydrolysis Method. See Part II, H7.

For the identification of the oil or oils, determine the refractive index, specific gravity, and iodine number and test for cottonseed oil and other oils (Part II, B2) after removal of the oil by naphtha extraction as under Sugars below.

Acidity

Dilute 15 g. of the sample with 200 ml. of water and titrate with *0.1 N alkali*, using *phenolphthalein indicator*.

Phosphoric Acid

Determine the total phosphoric acid as directed for eggs (Part II, H7).

Sugars

Process. *Removal of Oil.* Extract the oil from 20 g. of the sample by shaking with 80 ml. of *naphtha*, centrifuging, and removing the solvent layer with a pipet. Repeat 4 times or until the oil is removed as indicated by the color. Reserve the naphtha solution for the identification of the oil above.

Clarification. Remove the naphtha from the extracted residue by aspiration and transfer to a 100-ml. volumetric flask. Add 5 to 10 ml. of *5% phosphoric acid solution*, mix, dilute to volume, and filter.

Copper Reduction before Inversion. Pipet 80 ml. of the filtrate into a 100-ml. flask, neutralize to *phenolphthalein* with *1 + 1 sodium hydroxide solution*, cool, dilute to the mark, and determine reducing sugars by the Munson and Walker Method in 50 ml. (Part I,

Copper Reduction after Inversion. Invert another aliquot and again determine the reducing sugars, deduct that found before inversion, and obtain the sucrose by the factor 0.95.

Note. If starch is present and interferes with the clarification, remove the oil by the Röse-Gottlieb-Patrick Method (Part II, G1), using 1 ml. of *ammonium hydroxide* and 5 ml. of *ethanol* for each gram of the sample, and determine the reducing matter as directed in Part I, C6*a*.

Starch

A modification of the Sullivan calcium chloride-iodine-iodide copper reduction method (Part I, C6*a*) is worthy of trial.

Egg Constituents

$$\% \text{ of yolk} = 75.69P - 1.802N$$

$$\% \text{ of white} = 60.80N - 114.59P$$

$$\% \text{ of total egg} = \% \text{ of yolk} + \% \text{ of white}$$

in which P is the per cent of total phosphoric acid and N is the per cent of total nitrogen.

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C. VEGETABLE FOODS

Most of the succulent foods classed as vegetables are characterized by the absence of distinct acidity, sweetness, or fruity flavor. Those, however, of the melon group are distinctly sweet, but only faintly acid, and those of the tomato group are distinctly acid, but not very sweet.

Vegetables on drying at low temperatures retain their chemical characteristics but on canning starch is gelatinized. Aromatic seeds of the parsley family and leaves of the mint family, although grown in the vegetable garden, in dried form are classed as spices. As here treated Vegetable Foods includes both vegetables and their products.

Microscopic Structure. (Figs. 113 to 124). Being derived from different parts of the plant and from widely different species, the cell forms and cell contents are highly diversified.

Of the root and tuber vegetables, potatoes and sweet potatoes contain an abundance of characteristic large starch grains, whereas the taros and yams contain minute starch grains; salsify contains inulin and latex, but no starch; carrots contain starch and beautiful carotene crystals. Turnips and rutabagas may be starchy or starchless according to the variety and the season; they are rich in the woody elements of the fibro-vascular bundles. Beets show a remarkable development of concentric cambium layers.

Leaf vegetables, except when blanched, are crowded with chlorophyl grains; they are distinguished by the epidermal tissues, often with characteristic hairs.

Green peas and beans, as well as sweet corn, show immature elements of the ripe seed or grain. The common solanaceous fruit-vegetables (tomato, peppers) and especially cucurbitaceous fruits (squash, melon, cucumber) have seeds of highly complicated structure. Variety of structure in vegetables is almost as endless as the species and varieties the world affords.

Leguminous seeds (shown in the first page of cuts) are grouped as starchy (most legumes) with more or less kidney-shaped starch grains, and non-starchy (soy bean). They are distinguished by the size and relative height of the palisade-like cells of the outer epiderm and the more or less hourglass-shaped cells of the subepiderm as seen in cross sections.

The technique for the examination of dried, non-oily leguminous seeds is practically the same as that for cereal foods. Cross sections cut with a Gillette safety razor blade are of particular value in distinguishing the different species by the characteristics of the epidermal and subepidermal cells, although in grinding and macerating the cells often are isolated and lie in the same position as in cross sections. In the parts about the slit at the point of attachment (hilum) of the seed, the cells of both the epiderm and subepiderm are in several layers and are variously modified.

LEGENDS OF ILLUSTRATIONS ON THE FACING PAGE

Of the eight leguminous seeds illustrated, the soy bean, the peanut, and the yellow lupine, being rich in oil and protein, may be classed with the oil seeds; of the three only the peanut contains starch, the content of which, however, is only about one-fifth that in peas and beans. Note in the cross sections the variation in height and other characters of the epidermal palisade cells and the subepidermal, usually hourglass-shaped, cells. The magnification is ×160.

FIG. 113. Soy Bean. Left, seed in cross section: *S* seed coat, *E* endosperm, and *C* cotyledon with aleurone grains. Right, elements in surface view: seed coat with *pal* palisade cells, sub^1 outer and sub^2 deep focus of subepiderm (sub^3 on side), and *p* spongy parenchyma; *E* endosperm; and *C* outer epiderm of cotyledon.

The hourglass-shaped subepidermal cells are nearly as high as the palisade cells; the coloring matter characterizing the seeds is located in the latter.

FIG. 114. Peanut. Elements in surface view. Fruit coat: *h* pitted cell of hypoderm and *f* fiber. Seed coat: aep^2 outer epiderm (aep^1 in cross section), p^1, p^2, and p^3 parenchyma, and *iep* inner epiderm. *E* endosperm. Cotyledon: *ep* epiderm with stoma and *mes* mesophyl with starch grains and aleurone grains.

The outer epidermal (palisade) cells are nearly as broad as they are high and the subepidermal cells are not hourglass-shaped. Only one of the many curious forms of stone cells of peanut shells is pictured; others are L- or T-shaped, serving as trusses. The starch grains are round, of medium size.

FIG. 115. Common Bean. Left, seed in cross section: *S* seed coat and *C* cotyledon with *am* starch grains. Right, elements in surface view: seed coat with pal^1 outer and pal^2 deep focus of palisade cells, *sub* subepiderm, p^1, p^2, and p^3 spongy parenchyma, and *iep* inner epiderm; and *C* outer epiderm of cotyledon.

The subepidermal cells are not hourglass-shaped and further differ from the type in that each contains a monoclinic crystal of calcium oxalate. The starch grains are oval or kidney-shaped with a marked branching hilum.

FIG. 116. Smooth Pea. Left, seed in cross section, and right, elements in surface view. Seed coat: *pal* palisade cells, *sub* subepidermal cells, and *p* parenchyma. Cotyledon: *ep* epiderm and *mes* mesophyl with *am* starch grains.

The subepidermal cells, as brought out in the illustration, are strikingly hourglass-shaped with ribs. The hilum of the starch grains is seldom evident.

FIG. 117. Left, Lima Bean, and right, Yellow Lupine, in cross section. Seed coat with *pal* palisade cells, *sub* subepiderm, and *p* parenchyma. Cotyledon: mesophyl with *am* starch grains or *al* aleurone grains.

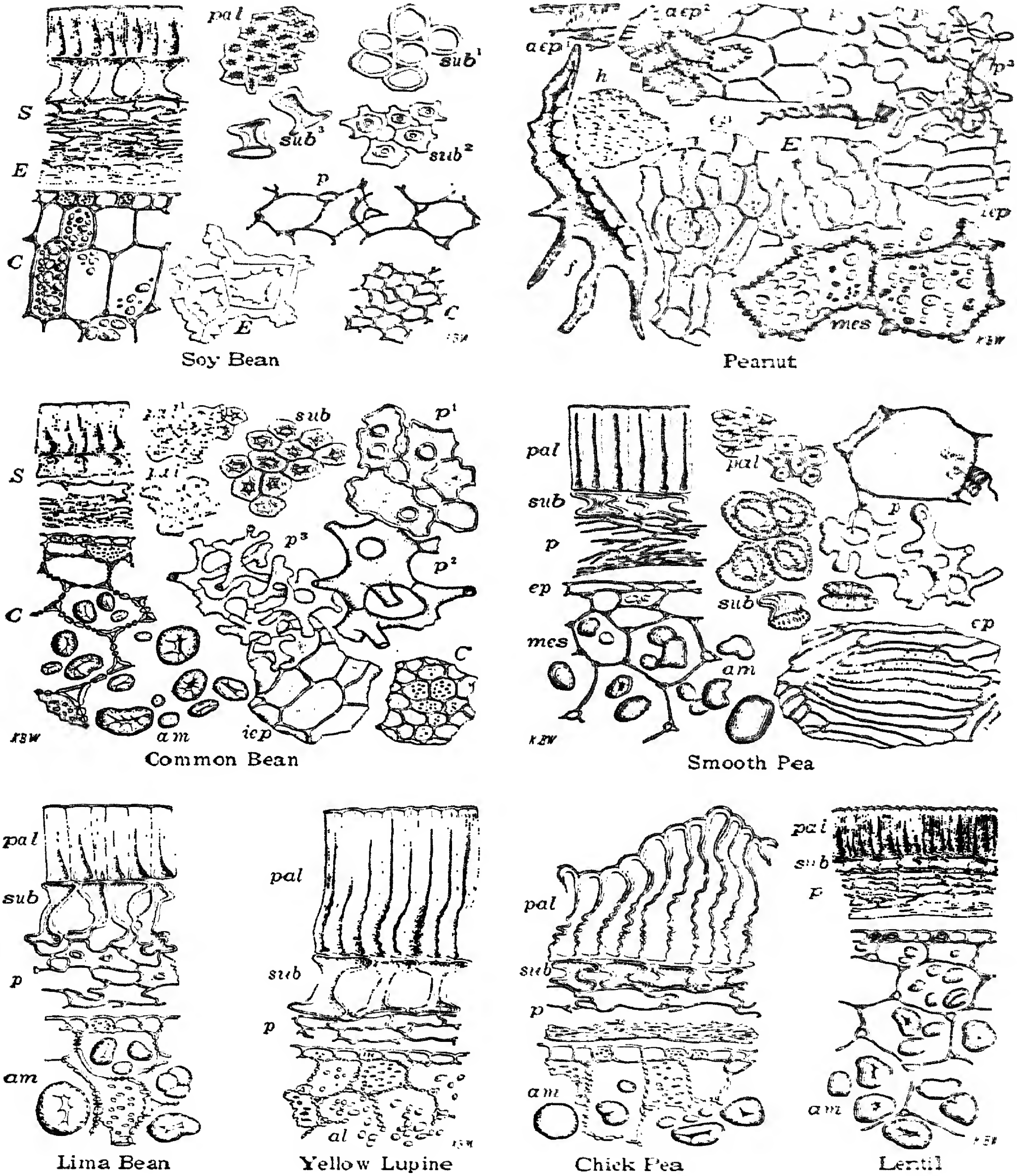
The subepidermal cells of the Lima bean are often as high as the palisade cells; distorted, as well as hourglass, forms are present.

The robust development of the epidermal and subepidermal layers of the yellow lupine is striking, the former being twice as high as the latter. Note the aleurone grains and the absence of starch.

FIG. 118. Left, Chick Pea, and right, Lentil, in cross section. Seed coat: *pal* palisade cells, *sub* subepiderm, and *p* parenchyma. Cotyledon: mesophyl with *am* starch grains.

The irregular height and wavy walls of the palisade cells distinguish the chick pea from the other species here figured.

The palisade cells of the lentil are narrow but of about the same height as those of the common bean; the hourglass cells are small. The starch closely resembles that of the common bean.


Soy Bean
Peanut
Common Bean
Smooth Pea
Lima Bean
Yellow Lupine
Chick Pea
Lentil

FIGS. 113–118.

LEGENDS OF ILLUSTRATIONS ON THE FACING PAGE

The six vegetables here shown illustrate those derived from root, tuber, leaf, and fruit; seed vegetables appear above. The magnification is ×160.

FIG. 119. Potato. *su* cork cells in surface view. *cr* cortex crystals. *P* phloem in longitudinal section with sieve tubes, companion cells, and starch parenchyma, *v* xylem vessels, and *p* pith with *am* starch grains.

The cork cells of the skin, the oval, medium-sized to very large, starch grains, and the lace-like vessels forming a tangle after removal of the starch by boiling with acid are the conspicuous elements. The aleurone grains, or rather crystals, found after careful search, represent the nitrogenous matter.

FIG. 120. Carrot. Elements in longitudinal section. *su* cork cells. *C* cortex with carotene crystals and starch grains. *Ph* phloem: *ol* oil and *am* starch grains. *f* substitute fibers. Xylem: *cm* cambium, *v* vessels, and *p* parenchyma.

Broad reticulated and spiral vessels characterize this and other root vegetables. The essential oil cavities, flanked by cells containing minute starch grains, are seen in carefully cut sections. Carotene crystals give the cortex its deep orange-red color. White carrots contain no carotene. The parsnip has similar cellular tissues.

FIG. 121. Sweet Potato. *su* cork cells in surface view. Elements in longitudinal section: *v* vessels, *l* latex vessel, *p* starch parenchyma, *am* starch grains, *s* sieve tubes, *c* companion cells, *cm* cambium, and *m* medullary ray.

Cork cells and large pitted vessels are conspicuous after boiling with dilute hydrochloric acid. The starch grains and the contents of the latex tubes are seen in sections mounted in water.

FIG. 122. Tomato. Fruit coat: *epi* epicarp and *hy* hypoderm in surface view. Seed coat: ep^1 outer epiderm, with false hairs, seen from below, and ep^2 in cross section. *E* endosperm and *R* radicle in cross section.

Delicately beaded cells make up the epicarp and hypoderm of the fruit. Lycopene crystals may be seen in the fruit on careful focusing. The false hairs of the seed are ribs of the epidermal cells.

FIG. 123. Lettuce. *aep* outer epiderm and *iep* inner epiderm with hairs, emergence, and stomata in surface view. Fibrovascular bundle of midrib in longitudinal section: *l* latex vessel, *p* parenchyma, *col* collenchyma, *c* companion cells, *s* sieve tube, *an* annular vessel, *sp* spiral vessel, *ret* reticulated vessel, and *sc* sclariform vessel.

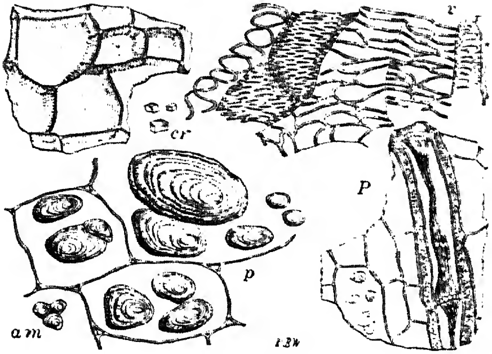
Jointed hairs (often with glandular apical cells) and multicellular emergences are characteristic of the epiderm. The various elements of the ribs and veins are not distinguishable from those of other allied leaf vegetables.

Epidermal tissues of the cabbage group (not illustrated) are without hairs and rather characterless.

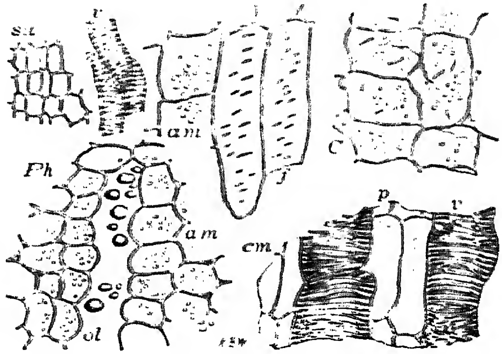
FIG. 124. Spinach. Elements in surface view: *iep* lower epiderm with capitate and pointed hairs and stoma, *mes* mesophyl with rosette crystal and chlorophyl grains.

Note the thick-walled pointed hairs and jointed hairs with large glandular tips, also the large oxalate crystal and chlorophyl grains in the mesophyl.

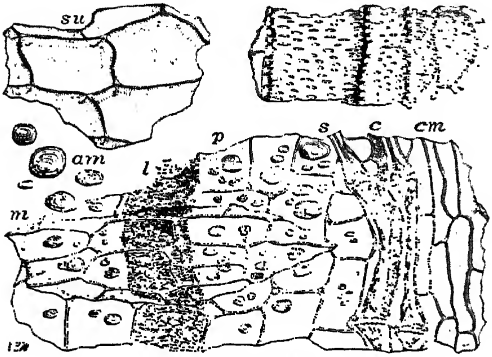
The epidermal cells of New Zealand spinach, belonging to another botanical family, bear numerous bladder-like hairs.



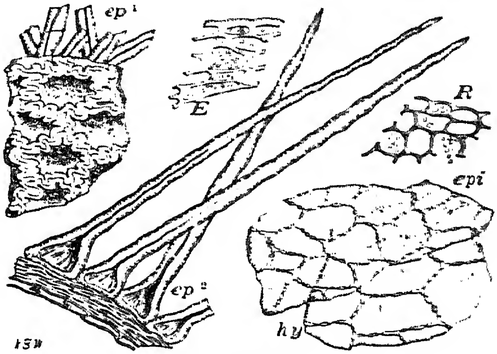
Potato



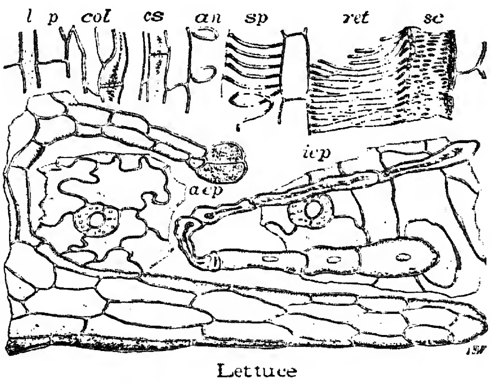
Carrot



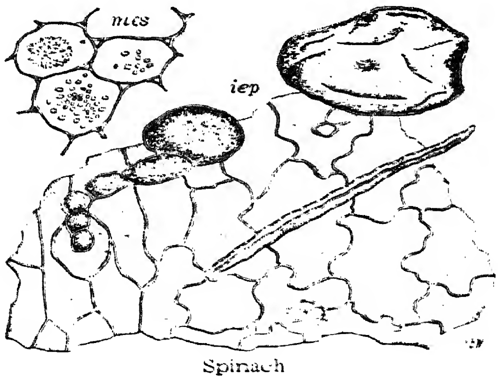
Sweet Potato



Tomato



Lettuce



Spinach

Figs. 119-124.

AVERAGE COMPOSITION OF VEGETABLES

	Water	Protein	Fat	Nifext	Fiber	Ash
	%	%	%	%	%	%
Fungus						
Field mushroom	91.80	4.83	0.31	2.04 †	....	1.02
Roots						
Carrot	89.0	1.0	0.1	7.9	1.0	1.0
Garden beet	88.47	1.53	0.14	7.94	0.88	1.04
Parsnip	80.0	2.2	0.4	14.8	1.3	1.3
Rutabaga	89.0	1.2	0.2	7.2	1.3	1.1
Root tuber						
Sweet potato *	71.77	1.36	0.28	24.34	1.23	1.02
Tubers						
Potato	78.89	2.14	0.10	17.36	0.56	0.95
Yam	78.23	1.11	0.12	18.60	0.96	0.98
Leaves and stems						
Asparagus	93.96	1.83	0.25	2.55	0.74	0.67
Cabbage	91.5	1.6	0.3	5.6 †	1.1	1.0
Celery	94.5	1.1	0.1	3.3 †	....	1.0
Chard	92.17	1.45	0.18	3.09	0.87	2.24
Kale ‡	79.69	2.77	0.99	13.43	1.63	1.49
Kohlrabi	90.1	2.0	0.1	5.5	1.3	1.3
Lettuce	86.28	2.27	0.95	6.22	2.57	1.71
Onion (white)	85.26	2.28	0.22	10.80	0.76	0.68
Spinach	92.3	2.1	0.3	3.2 †	0.9	2.1
Flowers						
Artichoke	85.5	2.8	1.4	6.8 §	2.4	1.1
Broccoli ‖	88.1	4.39	0.74	...	1.42	1.69
Cauliflower	90.82	1.62	0.79	4.94	1.02	0.81
Fruits and seeds						
Bean, string	89.2	2.3	0.3	7.4 †	1.9	0.8
" Lima	10.4	18.1	1.5	65.9 †	....	4.1
" navy	12.6	22.5	1.8	59.6 †	4.4	3.5
Cucumber	95.4	0.8	0.2	3.1 †	0.7	0.5
Egg plant	92.90	1.2	0.3	4.30	0.8	0.5
Garden pepper	88.0	1.6	2.1	5.6	1.9	0.8
Lentil	8.4	25.7	1.0	59.2 †	....	5.7
Okra	80.74	4.15	0.42	12.12	1.15	1.41
Peas, green	74.6	7.0	0.5	16.9 †	....	1.0
" dried	9.5	24.6	1.0	62.0 †	4.5	2.9
Pumpkin	87.53	1.92	1.49	6.25	1.84	0.96
Sweet corn	75.4	3.1	1.1	19.7 †	0.5	0.7
Tomato ¶	93.93	10.90	0.05	....	0.37	0.69

* Sugars as sucrose 2.48%. † Includes fiber. ‡ Sugars 0.72%. § Reducing sugars 0.6, inulin 2.5, and insoluble carbohydrates 0.6%. ‖ Starch 1.30 and pentosans 0.91%. ¶ Amides 0.40, acid as citric 0.49, dextrose 1.12, levulose 1.13, and sucrose 1.73%.

Chemical Composition. Although vegetables, as shown by the analyses in the table herewith, contain the same six groups of constituents (see Part I, C2) as the cereals and oil seeds, they differ greatly as to the nature and amount of individual members of the group on the dry basis and still more, because of the wide range of moisture content, on the fresh basis. These differences in composition are not due wholly to the parts of the plant which they represent.

Starch may or may not be present in root vegetables and in seed vegetables. There are saccharine and non-saccharine, acid and non-acid fruit vegetables. Leaf vegetables supply ascorbic acid and also contain more or less carotene. Garden peppers and tomatoes are particularly rich in vitamin A or its provitamin carotene. Roots of the composite family, notably salsify and the Japanese gobo, contain no starch, but abound in inulin and latex. Umbelliferous root vegetables (carrots, parsnips, and celeriac) and parsley leaves contain essential oil. Pepper-grass leaves, also radish and horse-radish roots, contain pungent sulfur compounds.

Our knowledge of the kind and amount of the constituents forming a large part of many vegetables is exceptionally limited. It is a field for study.

1. VEGETABLES

Sample

Dried peas, beans, and other dried legumes are ground in the same manner as cereals. Some succulent vegetables are pulped, expressed, and the juice and marc are separated for analysis; others are analyzed without separation into the parts. Whether or not the whole sample, or the marc, is dried at a moderate heat, then ground for analysis, depends on the material and the constituents to be determined. The same general rule applies to canned vegetables. After weighing, the contents of the can, peas, string beans, shelled beans, asparagus, spinach, beets, and some other vegetables are separated into solid and liquid portions; pumpkin, sweet potatoes, and other vegetables of uniform consistency are simply mixed well for analysis.

Protein; Fat; Organic Acids; Sugars; Fiber; Ash

The methods are partly those given in Part I, C1 to C13, and Part II, D2. The widely differing physical characters of vegetables, however, necessitate modification to meet the particular cases. Only methods specially designed for vegetables are given herewith.

Ammonia and Amide Nitrogen

Pucher, Vickery, and Leavenworth Distillation Colorimetric Method.[1] Apparatus. *Pucher, Vickery, and Leavenworth Distillation Assembly* (Fig. 125).

Pulfrich Photometer, provided with light filter S 43.

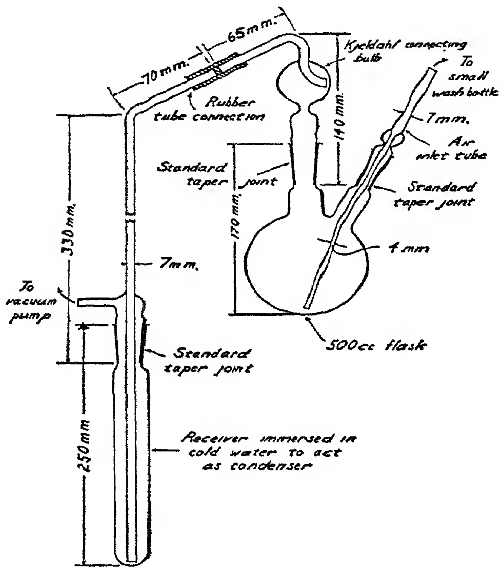
Reagents. *Phosphate-Borax Buffer Solution*, pH 6.5. Mix 750 ml. of 0.1 *M* KH_2PO_4 (13.6 g. per liter) and 250 ml. of 0.05 *M* $Na_2B_4O_7 \cdot 10H_2O$ (19.1 g. per liter).

Borax Solution, 5% in ammonia-free 0.5 *N* NaOH.

Nessler Reagent (Koch and McMeekin).[2] Dissolve 150 g. of KI and 112.5 g. of iodine in 100 ml. of water, then dissolve in the mixture 150 g. of mercury with shaking and cooling and pour slowly with stirring into 4875 ml. of 10% NaOH.

A. Ammonia Nitrogen Process. *Extraction*. Cut with scissors into thin slices 200 to 300 g. of the fresh plant tissue, avoiding loss of sap, press down into a beaker, cover with *ether*, and allow to stand until entirely flaccid (20 to 30 minutes). Strain the ether together with the aqueous fluid that

has exuded through cheese-cloth into a separatory funnel, draw the aqueous layer into a 1-liter flask, rinse the ether twice with 50-ml. portions of water, and add to the washings in the flask. Fold the tissue in cheese-cloth, envelop in press-cloth, and press the dry cake in the hydraulic press. Disintegrate by



Courtesy of Ind. Eng. Chem., Anal. Ed. 1935, 7, 153

FIG. 125. Pucher, Vickery, and Leavenworth Distillation Assembly

hand, mix with water, and again press. Repeat this process once more, add the extracts, together with rinsings of the cloths and apparatus, to the flask and dilute to volume.

Ammonia Distillation. Mix in the distilling flask a suitable aliquot (usually 25 ml.) of the fresh tissue extract (or 0.1 to 0.5 g. of a finely ground dry tissue and 20 ml. of water) with 10 ml. of *phosphate-borax buffer solution.* Charge the receiver with 3 ml. of *0.1 N hydrochloric acid,* lubricating the joints with *vaseline,* connect the flask with the condenser and receiver, and just before connecting the air-inlet tube, introduce 3 ml. of *alkaline borax solution.* Exhaust the air and adjust the air inlet to a flow of 2 or 3 bubbles per second. Raise the bath, containing water at 40 to 42°, so as to immerse the entire flask and distil for 15 minutes, maintaining the same temperature. Lower the bath, admit air through the inlet, break the rubber connection, and disconnect the suction. Rinse the vapor tube and transfer the distillate to a 25 x 200 mm. test tube graduated at 50 ml. or a 50-ml. volumetric flask. Add water to about 35 ml. and 5 ml. of *Nessler reagent* and dilute to volume.

Color Measurement. Determine the extinction coefficient in the Pulfrich photometer, using light filter S 43. Find the milligrams of nitrogen corresponding to the extinction coefficient on a curve plotted for nesslerized 0.05 to 2.0 ml. of ammonia nitrogen in 50 ml. against the corresponding extinction coefficients.

NOTE. Tissues should be dried at a temperature below 70°; even with these precautions, loss or changes of the nitrogenous constituents may take place.

B. AMIDE NITROGEN PROCESS. Mix a 5-ml. aliquot of the water extract of the dried sample with 1 ml. of *6 N sulfuric acid* in a 25 x 200 ml. test tube and close with a stopper that carries a short length of 1-mm. capillary tubing. Heat the tube for 3 hours in a boiling water bath, transfer the contents to the distilling apparatus with 20 ml. of water, add 5 ml. of *1.0 N sodium hydroxide solution* to neutralize most of the acid, then add 5 ml. of *alkaline borax solution,* distil, and determine the ammonia as above.

Correct the result for the apparatus blank and for "preformed" ammonia determined directly on the sample of the dried tissue. More trustworthy results probably may be obtained by the ether cytolysis method, but the greatest care must be taken to avoid sampling error.

Glutamine

See also Part I, C4c.

Pucher and Vickery Pyrrolidone Carboxylic Acid Manometric Method.[3] The method is not a substitute for the amide hydrolysis method, but a more specific means of estimating small amounts of glutamine, especially in the presence of large amounts of asparagine or other substances that might interfere with the simpler hydrolytic method.

Apparatus. *Pucher and Vickery modification of the Widmark Extractor,*[4] consisting of two Erlenmeyer flasks connected side by side with a short length of wide-bore tubing sealed into the sloping walls of the flasks, the whole being rocked through an angle of ±15° around an axis placed beneath the horizontal connecting tube. A plant extract is placed in one flask and sodium bicarbonate solution in the other, then the solvent is added to the level of the connecting tube.

Van Slyke Manometric Apparatus.

Reagents. *Buffer Solution,* pH 8.5. Dissolve 4.957 g. of KH_2PO_4 and 12.167 g. of $Na_2B_4O_7 \cdot 10H_2O$ in water and dilute to 1 liter.

Ethyl Acetate, 95 to 99% by volume. One milliliter shaken with 10 ml. of water, titrated at once, must not require more than 0.1 ml. of 0.1 *N* NaOH solution to give a faint pink color with phenolphthalein. Recover after use by washing 1 liter twice with 200 ml. of 10% Na_2CO_3 solution followed by water until neutral. Dry over $CaCl_2$ and distil.

Process. *Extraction.* Mix 5 g. of the dry powdered tissue with 80 ml. of water and heat at 80° for 10 minutes with careful stirring. Cool the suspension at once, transfer to a centrifuge tube graduated at 100 ml., dilute to the mark, mix, and centrifuge. Decant the clear liquid through a plug of glass wool in a dry funnel. Preserved by *toluene* for several months, the extract is suitable for analysis notwithstanding considerable amide hydrolysis.

Hydrolysis. Place 10 ml., or other suitable aliquot, in a 200 x 25 mm. test tube and add 10 ml. of the pH *8.5 buffer solution.* The resulting pH should be about 6.5. Stopper lightly and heat in a boiling water bath for 2 hours, ignoring loss of ammonia.

Pyrrolidone Carboxylic Acid Extraction. Transfer the solution to one of the 500-ml. flasks of the Widmark extractor, rinsing with 10 ml. of *20% sodium sulfate solution* and adjusting the reaction to pH 2.4 ± 0.2 by the addition of (usually 1.5 ml.) *1.2 N sulfuric acid* and dilute to 40 ml. with water. Charge the other flask of the apparatus with 40 ml. of *0.5 N sodium bicarbonate solution,* add sufficient *ethyl acetate,* stopper, and oscillate at the rate of about 144 cycles per hour for 18 hours. The alkaline solution thus obtained contains the whole of the pyrrolidone carboxylic acid derived from the glutamine of the sample, together with most of the malic, citric, and other organic acids, traces of amino acids, and other substances. Draw off the alkaline solution from beneath the solvent by a fine-bore pipet attached to a distilling flask and a suitable source of suction. Wash the solvent by gentle agitation with two 5-ml. portions of water. It is usually advisable to sample the solution on the acid side of the extractor for a confirmatory test of the reaction by a glass electrode. Neutralize the alkaline solution with *4 N sulfuric acid* to a few drops of *bromophenol blue indicator,* evaporate rapidly *in vacuo* to a volume of about 10 ml., transfer to a 50-ml. volumetric flask, and dilute to volume.

Pyrrolidone Carboxylic Acid Hydrolysis. Transfer a 15-ml. aliquot of the neutralized solution to a 50-ml. Erlenmeyer flask, add 3 ml. of *hydrochloric acid,* together with a few angular fragments of *quartz,* cover with a small glass bulb, and heat in a boiling water bath or reflux on a hot plate for 2 hours. Evaporate the solution on a hot plate to

about 5 ml. (about 15 minutes). Add a drop of *bromophenol blue indicator*, make the solution distinctly alkaline with *10 N sodium hydroxide solution*, and then acidify with 1 ml. of *glacial acetic acid*. Transfer the solution to a test tube graduated at 15 ml. and dilute to the mark, thus restoring to the original volume of the aliquot before hydrolysis.

Determine the amino nitrogen in 5-ml. portions of this solution, and also in suitable aliquots of the solution before hydrolysis, in the manometric Van Slyke apparatus.

CALCULATION. Multiply the increase in amino nitrogen brought about by hydrolysis (equivalent to the glutamine amide nitrogen of the original sample) by 2000, thus obtaining the percentage of glutamine amide nitrogen in the dried tissue.

EXAMPLES. Rhubarb petiole (pH 1.9) and rhubarb leaf (pH 1.9), above method, 0.113 and 0.490; nitrogen amide hydrolysis method, 0.118 and 0.450% of glutamine amide nitrogen respectively.

CHOLINE AND BETAINE

Reifer Periodate Volumetric Micro Method.[5] Choline is determined by precipitation of the periodate in neutral solution followed by titration of the periodate with thiosulfate; choline and betaine are then precipitated together in an acid solution and determined together by titration.

REAGENTS. *Triiodide Reagent.* Dissolve 2.5 g. of iodine, 3.75 g. of KI, and 10 g. of NaCl in 100 ml. of water.

Aluminum Hydroxide Suspension. Dissolve 10 g. of alum in 150 ml. of water and add 100 ml. of 0.2 *N* NaOH.

PROCESS. A. CHOLINE. *Precipitation as Periodate.* Treat 3 ml. of the extract of the sample (containing 0.1 to 5.0 mg. of choline) at below 10° with 5 ml. of the *triiodide reagent.* After allowing to stand 3 hours, centrifuge for 2 minutes and add 0.5 ml. of *aluminum hydroxide suspension* to form a coating. Centrifuge at 2500 r.p.m. for 5 minutes and wash the walls of the tube with 3 ml. of *10% sodium chloride solution* at 0°. Remove the sodium chloride solution without centrifuging and dissolve the aluminum hydroxide in 2 ml. of *5% sulfuric acid* without disturbing the precipitate. Centrifuge for 3 minutes and carefully decant the supernatant liquid.

Titration. Dissolve the precipitate in 1 to 3 ml. of *ethanol* and titrate with *standard sodium thiosulfate solution.*

B. CHOLINE PLUS BETAINE. *Periodate Precipitation.* Treat 3 ml. of the solution of the sample in a 25-ml. centrifuge tube with 0.3 g. of *sodium chloride*, 0.5 ml. of *sirupy phosphoric acid*, and 1 ml. of the *triiodide reagent*, but add no aluminum hydroxide suspension. Keep for 3 hours in a salt-ice bath at −5 to 10°, then centrifuge for 5 minutes, thus obtaining a separation in three layers. Remove by suction the potassium triiodide forming the top layer and wash the sides of the tubes three times with 3-ml. portions of ice water without centrifuging, then remove the middle layer of phosphoric acid in a similar manner.

Titration. Treat the precipitate of periodate forming the bottom layer as described above for the choline precipitate.

NOTE. Dimethylamine, trimethylamine, certain cyclic bases, and many alkaloids also yield precipitates with potassium triiodide.

SOLANINE

Potatoes with an abnormally high solanine content have caused epidemics of poisoning in Germany;[6] even a normal content may affect the public health far more than is commonly recognized. It is well known that the outer layers of the tuber contain more than the inner and that boiling in the jackets tends to prevent removal. One of the present writers has definitely traced attacks of indigestion to immature potatoes boiled without

paring. During boiling, pared potatoes lose much of their solanine which, in the preparation of stews, remains in the broth and may be the cause of the indigestibility.

Meyer Ammonia Precipitation Gravimetric Method.[7] This method and the Wintgen modification are stated by the latter author to yield concordant results.

PROCESS. *Maceration, Expression, and Ethanol Extraction.* Remove the juice from the pulped sample by pressure, neutralize with *ammonium hydroxide*, evaporate nearly to dryness, and extract both the residue and the pomace with hot *ethanol.*

Precipitation. Evaporate nearly to dryness the combined ethanolic extracts, take up in water slightly acidulated with *sulfuric acid*, filter, wash, and supersaturate with *ammonium hydroxide.* Collect the solanine on a filter and wash thoroughly with water.

Purification. Dissolve the precipitate in *ethanol*, filter from impurities, evaporate the filtrate at a moderate heat, cool, and weigh.

I. Wintgen Modification.[8] The preliminary steps differ from those of the original method in the following details: (1) the pomace is thoroughly extracted in a percolator with cold *ethanol containing 0.5% acetic acid* and (2) the percolate is neutralized with *ammonium hydroxide* and added to the ethanolic extract of the concentrated juice. The remainder of the process is like that given above, beginning with Precipitation.

II. Bömer and Mattis Modification.[9] PROCESS. *Maceration, Expression, and Concentration.* Stir 200 to 300 g. of the grated sample for 30 minutes at room temperature with an equal volume of water and squeeze out the liquid in a linen bag. Repeat the treatment three times, adding, however, 5 ml. of *90% acetic acid* to the water used for each extraction. Add to the combined extracts *ammonium hydroxide* to slight alkaline reaction, then add 10 g. of *kieselguhr* and evaporate on a water bath, stirring well as the mass approaches dryness. Finally grind the dry residue to a powder.

Ethanol Extraction. Take up the residue in boiling *ethanol*, then either (1) extract in a continuous apparatus with *ethanol* for 10 hours, interrupting the process after 5 hours to stir the residue, or (2) reflux with 3 or 4 portions of 100 to 125 ml. of *ethanol*, each for one-half hour, and filter.

Precipitation. Distil off the ethanol, dissolve the residue in 50 to 100 ml. of water containing 3 to 5 drops of *glacial acetic acid*, add *ammonium hydroxide* to slight alkaline reaction, and heat for 30 minutes on a water bath, thus precipitating the solanine. Collect the precipitate on a filter and wash with *2.5% ammonium hydroxide.*

Reprecipitation. Dissolve the precipitate in 25 ml. of warm *ethanol*, filter, distil the ethanol, dissolve the residue in 50 to 100 ml. of acidified water, and precipitate with *ammonium hydroxide* as before, then filter on a weighed paper, wash, dry at 100 to 105°, and weigh.

Correction. Add to the weight of the precipitate 2.75 mg. for each 100 ml. of liquid used in the precipitation.

EXAMPLES. Normal tubers 20 to 89, abnormal tubers causing poisoning 277 to 583 γ/g.

III. Schowalter and Hartmann Modification.[10] These authors, unlike Wintgen, do not use *acetic acid* in the extraction but add 0.5 ml. per liter to the extract for the precipitation of the proteins. After filtrating and neutralizing with *ammonium hydroxide*, they evaporate to a thin sirup, acidify with *acetic acid*, and extract with hot *ethanol*. Purification is carried out by repeated solution in *acetic acid*, precipitation with *ammonium hydroxide*, and solution in *ethanol*. Finally the ammonium hydroxide precipitate is collected on a weighed filter, dried, and weighed.

EXAMPLES. During storage, the solanine content of potato parings increased from 99.5 to 243.2; that of the interior decreased from 225.5 to 172.8 γ/g.

SUGARS

Hoffman Ferricyanide Colorimetric Method.[11] The method was designed originally for the determination of dextrose in blood and urine.

I. Forsee Photometric Modification.[12] The change in color of ferricyanide to colorless in passing to ferrocyanide is utilized in this procedure originating at the University of Florida Experiment Station.

APPARATUS. *Photoelectric Colorimeter,* Cenco-Sheard-Sanford photelometer with a blue filter and 12 ml. absorption cells. Calibrate as follows. Place 2 ml. of solutions containing 0 to 0.4 mg. of pure dextrose in test tubes or centrifuge tubes marked for 15 ml. and add exactly 3 ml. of alkaline ferricyanide reagent to each. Immerse the tubes in boiling water for 5 minutes, cool under the tap, dilute to the mark, and mix. Determine the color intensity against distilled water set at 100, using a blue filter. Plot the micro ammeter readings against milligrams of dextrose on semi-logarithmic paper. This standard curve remains unchanged after 5 months.

REAGENT. *Alkaline Ferricyanide Reagent.* Dissolve 1.8 g. of $K_3Fe(CN)_6$, purified according to Peters and Van Slyke,[13] and 40 g. of anhydrous Na_2CO_3 in water and dilute to 1 liter. Store in an amber-colored bottle in the dark. Make a blank reading with each series of determinations.

PROCESS. *Ethanol Extraction.* Treat a weighed portion of green or quick-dried plant sample in the usual manner with hot *80% ethanol.* Heat measured portions of plant juices in a boiling water bath to destroy enzymes. In either case clarify as directed by Hassid (See Hassid Modification for Reducing Sugars, Part I, C6*a*).

Clarification. Evaporate on a water bath to about 10 ml. a volume of the extract or juice, containing 5 to 35 mg. of reducing sugar, cool, add 5 ml. of *saturated normal lead acetate solution*, and remove the excess of lead with 10 ml. of *saturated disodium phosphate solution*, then add 0.3 g. of *Norite decolorizing charcoal* and allow to stand with frequent shaking for 30 minutes. Filter on a thin layer of *talc* in a Büchner funnel, as directed by Hassid,[14] and wash several times with a little water. Make up the solution to 100 or 200 ml. in a volumetric flask.

A. REDUCING SUGAR.

Color Formation. Pipet an aliquot of not more than 2 ml., containing 0.1 to 0.35 mg. of dextrose, into a 15-ml. centrifuge tube, dilute to 2 ml., and treat as directed above for the calibration of the standard dextrose solution.

Reading. Obtain the photoelectric colorimeter reading and read the weight in milligrams of dextrose in the aliquot directly from the calibration curve.

B. TOTAL SUGARS. *Inversion.* Place 50 ml. of the clarified solution in a 100-ml. volumetric flask, bring to the acid color of *methyl red* with *dilute acetic acid,* using an amount determined on a separate 5- or 10-ml. aliquot. Add 2 to 4 drops of *1% Wallerstein invertase scales solution* and allow to stand overnight at room temperature. Run a blank on the invertase solution simultaneously. Dilute the flasks to volume and determine reducing sugars on aliquots as directed above.

II. Morrell Modification,[15] As stated by Morrell, of the U. S. Dept. Agr., Bur. Plant Industry, Charleston, S. C., no essential changes from the Forsee modification have been made except as regards the volumes and concentration of the reagent, an increase in time of heating from 5 to 10 minutes, and the use of the Evelyn photoelectric colorimeter equipped with blue filter No. 420.

REAGENT. *Alkaline Ferricyanide Solution.* (1) Dissolve 40 g. each of $K_3Fe(CN)_6$ and sodium carbonate monohydrate in water and dilute to 2 liters. (2) Dissolve 800 g. of the carbonate in water and dilute to 2 liters. For use, pipet 50 ml. each of (1) and (2) into a

2-liter volumetric flask and dilute to the mark.

CALCULATION. The calculation curve is plotted with 0 to 1.3 mg. of sugar by 0.1-mg. steps as abscissas and photometric density from 0 to 1.0 by 0.1 steps as ordinates.

STARCH

Märcker Diastase Method. (See Part I, C6*a*.) Balch and Phillips,[16] in the determination of starch in sweet potatoes, obtained higher results than the factory yield warranted. This is apparently due to water-insoluble substances that are calculated with the starch. By treatment of the pulp with *0.02 or 0.04 N calcium or barium hydroxide solution*, results consistent with the yield were obtained.

INULIN

Strepkov Phosphomolybdic-Permanganate Volumetric Method.[17] The method, as employed at Samarkand University, U.S.S.R., depends on the formation with the Folin and Wu reagent of a blue color which is discharged by titration with permanganate solution.

REAGENT. *Folin and Wu Reagent.* Mix equal volumes of 10% $Na_2WO_4 \cdot 2H_2O$ and 0.66 N H_2SO_4.

PROCESS. *Extraction and Clarification.* Treat 1 g. of the comminuted sample with *ethanol* to remove soluble glucides, then extract three times with 20 to 25 ml. of water at 40 to 45°. Add *normal lead acetate solution* and *disodium phosphate solution*, mix, filter, wash, and dilute the filtrate to 100 ml. in a volumetric flask.

Phosphomolybdic Treatment. Pipet 2 ml. of the filtrate into a test tube, add 2 ml. of *Folin and Wu reagent*, and heat in a boiling water bath 1.5 hours.

Titration. Cool and titrate with *standard 0.01 N permanganate solution* delivered from a micro buret to the appearance of a faint rose color. If the inulin solution is of high concentration, the end-point color is faint yellow.

CALCULATION. Use the formula: 1 mg. of inulin = 1.85 ml. of 0.01 N permanganate solution.

HYDROCYANIC ACID

(Cyanogenetic Glucosides)

The cyanogenetic glucosides linamarin of flaxseed and phaseolunatin of certain Asiatic varieties of the Lima bean (*Phaseolus lunatus*) are believed to be identical or isomeric. Early in the century their presence in human and animal foods caused widespread alarm, although there is no evidence that occidental beans contain any appreciable amount of the glucoside and linseed in the form of linseed meal appears to have lost in the process of extraction and drying any possible toxicity it may have once possessed.

Two methods for the determination of hydrocyanic acid formed in beans from cyanogenetic glucosides have been tentatively adopted by the A.O.A.C.

A. Modified Shûlek-Lang Acid Titration Method. See Part I, C6*b*, Hydrocyanic Acid.

PROCESS. Macerate in an 800-ml. Kjeldahl flask 10 to 20 g. of the sample, ground to pass a 20-mesh sieve, with 100 ml. of water for 2 hours. Add to the mixture 100 ml. of water and connect with a steam distillation assembly, the condenser tube of which has an extension that dips below a mixture of 20 ml. of *0.02 N silver nitrate solution* and 1 ml. of *nitric acid* in the receiver. When 150 ml. have passed over, filter the distillate through a Gooch crucible, rinse, and wash with water, then titrate the excess of silver nitrate with *0.02 N potassium thiocyanate solution*, using *ferric ammonium alum solution* as indicator; 1 ml. of 0.02 N silver nitrate solution = 0.54 mg. of hydrocyanic acid.

B. Alkaline Titration Method (Labatti Iodide-Silver Nitrate Method. See Part I, C6*b*.

PROCESS. Distil as under A, using 200 ml. of water, but collect the distillate in a solution of 0.5 g. of *sodium hydroxide* in 20 ml. of water. Make the distillate up to the mark in a 250-ml. volumetric flask and pipet 100 ml. into a beaker. Add 8 ml. of *6 N ammonium hydroxide* and 2 ml. of *5% potassium iodide solution* and titrate with *0.02 N silver nitrate solution* delivered from a micro buret. The end-point is a faint but permanent turbidity; 1 ml. of 0.02 *N* silver nitrate solution = 1.08 mg. of hydrocyanic acid.

CAROTENE

See also Part I, C10, and Part II, A1, A2, G1, and G7.

Guilbert Colorimetric Method.[18] The method, developed at the University of California, was designed for forage, but is well adapted for garden leaf vegetables. As originally described, it differs from the modification which follows chiefly in that the first extraction is made with *ether* and the final solution is compared with a solution containing 3.06 g. of *Naphthol yellow* and 0.45 g. of *Orange G* crystals in 1 liter.

Peterson and Hughes Modification.[19] APPARATUS. *Spectrophotometer, Photoelectric Colorimeter*, or *Colorimeter*.

PROCESS. *Ethanol-Alkali Digestion.* Weigh 1 to 5 g. of the sample into a 200-ml. Erlenmeyer flask and add for each gram 20 ml. of *saturated potassium hydroxide solution in ethanol.* Reflux for 30 minutes, heating on a steam bath or hot plate and washing down with *ethanol* any of the sample that collects on the sides of the flask.

Naphtha Extraction. Cool and filter through a sintered-glass funnel of No. 3 porosity into a 500-ml. separatory funnel. Extract the residue by shaking for about 1 minute each with small portions of *naphtha* until no more color is removed, using a total of about 100 ml. If the residue forms an adherent mass, break up by shaking with 10 to 15 ml. of *ethanol,* then extract with two or three portions of 20 ml. each of *naphtha.* Pour gently 100 ml. of water through the ethanol-naphtha solution in the separatory funnel. Draw off the alkaline ethanolic solution, reextract three times by gently shaking with 30 ml. of *naphtha,* using two other separatory funnels, combine the naphtha extracts, and wash free from alkali with 50-ml. portions of water (about ten times), testing with *phenolphthalein solution.*

Xanthophyl Removal by Methanol. Extract the naphtha solution with five to twelve portions of 25 ml. each of *90% methanol,* shaking after each addition for 2 minutes. Wash the naphtha solution twice with 50 ml. of water to remove the methanol. Evaporate under reduced pressure or dilute to secure a suitable concentration, filter through *anhydrous sodium sulfate* into a volumetric flask, and make up to the mark.

Color Measurement. A. By Spectrophotometer. Measure the optical density at wave lengths Å 4500, 4550, 4700, and 4800, using the extinction coefficients 243, 231, 207, and 212 for naphtha (b.p. 40 to 60°) and 238, . . ., 200, and 212 for Skelly Solve (b.p. 60 to 70°) respectively, and determine the carotene concentration for each wave length. Report the average to 0.1 γ/g.

B. By Colorimeter. Compare with *0.1% potassium dichromate solution.* Find the gammas per gram of carotene corresponding to the height of the dichromate column in centimeters in the accompanying table, then calculate the carotene in the sample (S) to 0.1 γ/g. by the formula

$$S = \frac{T \times M}{G \times D}$$

in which T is the gammas per gram from the table, M is the milliliters of solution, G is the

charge in grams, and D is the height in centimeters of the solution of the sample.

CAROTENE FROM HEIGHT OF DICHROMATE COLUMN (PETERSON AND HUGHES)

$K_2Cr_2O_7$ 0.1%	Carotene	$K_2Cr_2O_7$ 0.1%	Carotene
mm.	γ/g.	mm.	γ/g.
1.0	0.5	6.6	4.1
1.2	0.7	6.8	4.2
1.4	0.8	7.0	4.3
1.6	0.9	7.2	4.5
1.8	1.0	7.4	4.6
2.0	1.2	7.6	4.7
2.2	1.4	7.8	4.8
2.4	1.5	8.0	4.9
2.6	1.6	8.2	5.0
2.8	1.7	8.4	5.2
3.0	1.8	8.6	5.3
3.2	2.0	8.8	5.4
3.4	2.1	9.0	5.6
2.6	2.2	9.2	5.8
3.8	2.3	9.4	5.9
4.0	2.5	9.6	6.0
4.2	2.6	9.8	6.1
4.4	2.7	10.0	6.3
4.6	2.8	10.2	6.5
4.8	2.9	10.4	6.7
5.0	3.1	10.6	6.8
5.2	3.2	10.8	6.9
5.4	3.4	11.0	7.1
5.6	3.5	11.2	7.3
5.8	3.6	11.4	7.4
6.0	3.8	11.6	7.5
6.2	3.9	11.8	7.6
6.4	4.0	12.0	7.8

Hegsted, Porter, and Peterson Diacetone Spectrophotometric Method.[20] The distinctive feature of this method, devised at the University of Wisconsin, originally designed for silage but suited for various succulent vegetable products, is the substitution of 95% diacetone for 90% methanol or 85% ethanol for removal of the pigments other than carotene.

APPARATUS. *Spectrophotometer.*

REAGENT. *Diacetone.* acetone-free 95%. Dilute 100 volumes of acetone-free diacetone (Commercial Solvents Corp.) with 5 volumes of water.

PROCESS. *Ethanol Extraction and Saponification.* Mix and grind 0.5 to 1.0 kilo of fresh silage, remove 25 g. to a flask, add 200 ml. of *ethanol*, and reflux for 40 minutes. Decant the ethanolic extract, then add 200 ml. of *ethanol* to the residue, and reflux as before. To the combined extracts, add 40 ml. of *20% ethanolic potassium hydroxide solution*, shake, and allow to stand overnight.

If desired, follow the alternate procedure of refluxing the sample directly with *ethanolic potassium hydroxide solution*, maintaining the same volume (440 ml.) and ethanolic content of the first solution.

Naphtha Extraction. To the residue from the ethanolic extraction, add 80 ml. of *naphtha* (65 to 75°), heat just to boiling, and allow to cool, thus completing the extraction of carotene from the material.

Pipet 5 ml. of the naphtha extract and 25 ml. of the ethanolic extract (these being equivalent to the same amounts of silage), together with 2 ml. of *20% ethanolic potassium hydroxide solution*, into a separatory funnel, then add 15 ml. of *naphtha* and 7 or 8 ml. of water, and shake. After allowing to stand until the layers separate, draw off the ethanolic solution and reextract with two more 15-ml. portions of *naphtha*, thus removing all the carotene. The yellow color of the last portion of naphtha is due to xanthophyls.

Non-Carotene Pigments Removal with Diacetone. Wash the combined naphtha extracts in a separatory funnel with four 15-ml. portions of water, thus removing the alkali. To remove non-carotene pigments, extract the naphtha solution with four 10-ml. portions of *95% diacetone*, shaking vigorously after each

addition, and allowing to stand until the separation is complete, discarding the lower layer. Finally wash the carotene solution twice with water to remove the diacetone.

Spectrophotometric Reading. Make up the naphtha solution of carotene to 50 ml. with the same solvent and read the color value in the spectrophotometer.

CALCULATION. Report in terms of "apparent carotene" based on $E^{1\%}_{1\ \mathrm{cm.}}$ 480 mμ = 2150. Although a chromatographic analysis showed the presence in some cases of as high as 25% of non-carotene pigments, the originators of the method believe that the apparent carotene is a close approximation to the true amount, since there are losses in the process.

Kernohan Soda Ash Colorimetric Method.[21] In this recent method, designed for alfalfa but applicable to other forage plants and leaf vegetables, *soda ash* is substituted for magnesium oxide or calcium carbonate in the Tswett column. Only the following brief description is available.

Digest at room temperature overnight 1 g. of material with *naphtha* in a stoppered flask. Pour the extract on a column of finely divided *soda ash* and apply suction. When the solvent containing the carotene ceases to come through, wash with *naphtha* until the last portions are colorless. Collect the solution of the carotene and washings and determine the carotene content colorimetrically.

Fraps and Kemmerer Magnesium Hydroxide Adsorption Method.[22] The method, devised at the Texas Agricultural Experiment Station, applies to feeds, also doubtless to vegetables, the only carotenoids of which are carotene and xanthophyl. See Fraps and Kemmerer Chromatographic Method for the determination of carotenoids in corn (maize), Part II, A1.

APPARATUS. *Colorimeter.*

REAGENTS. *Activated Magnesium Hydroxide (Xanthophyl Reagent).* Activate 50 g. of light magnesium oxide by heating with 50 ml. of water in an evaporating dish on the water bath for 30 minutes. Shake 0.5 g. with 10 ml. of purified carotene solution (or 2.5 g. with 50 ml.) and allow to settle or separate by centrifuging. Read the color before and after the treatment. Treat a xanthophyl solution in the same manner. If none of the carotene and all of the xanthophyl is adsorbed, the reagent is suitable for use. If not, try another lot.

Standard Carotene Solution. Dissolve 0.1 g. of pure carotene in 2 ml. of chloroform, precipitate with about 25 ml. of methanol, collect on a filter, dry between filter paper and in a vacuum desiccator. Dissolve 20 mg. in a few drops of chloroform and dilute to 50 ml. with light naphtha, then dilute 5 ml. of the diluted solution to 1 liter with the same solvent.

Standard Xanthophyl. Saponify about 6 g. of alfalfa with 120 ml. of ethanolic KOH solution and extract with ether as directed by Fraps and Kemmerer.[23] After the ether has evaporated, take up the residue in light naphtha, and wash with 90% methanol until no more color is removed. Extract the methanol fraction twice with naphtha to remove traces of carotene, then extract this fraction with 90% methanol as directed above and add this extract to the original methanol extract. Remove the methanol with diminished pressure and take up the residue in 100 ml. of naphtha. Dry over anhydrous Na_2SO_4 and dilute to 2 γ/g.

PROCESS. Prepare the carotene solution in a suitable solvent and read the color. Estimate the crude carotene if desired. If necessary, adjust to 2 γ/g. of the color equivalent to carotene. Shake with the *magnesium hydroxide* in the proportion of 0.5 g. to 10 ml., filter, and again read the color. Report as gammas per gram of pure carotene.

Fraps, Meinke, and Kemmerer Modification.[24] Fraps, Kemmerer, and Greenberg[25] found that some lots of *magnesium oxide*, even though activated, adsorb a considerable

amount of carotene, but that *magnesium carbonate*, either activated or mixed with 10% of water, adsorbs xanthophyl completely without adsorbing carotene at all. Neither adsorbing agent, however, is employed in the present A.O.A.C. Method or the following modification for green vegetables and fodders.

The procedure for yellow corn is given in Part II, A1.

PROCESS. *Extraction.* Soak 100 g. of the sample in a large evaporating dish with 100 ml. of *95% ethanol* for 5 minutes, cut up with scissors, and grind with 100 g. of *white sand.*

If means are not at hand for immediate weighing, place the approximate amount in a tared jar containing a weighed amount of *ethanol,* then weigh on arrival, cut with scissors, and grind with *sand.*

Decant the liquid, strain through cheesecloth, make to a definite volume, and weigh the moist solids. Mix for analysis aliquots of the liquid and the moist solids equivalent to 5 g. of the fresh substance.

Saponification. Reflux for 30 minutes with 50 ml. of *12% ethanolic potassium hydroxide solution* and cool.

Ethanol-Naphtha Extraction. Add 50 ml. of *naphtha*, decant the liquid into a separatory funnel, grind the residue with 15 ml. of *naphtha* and then with a mixture of 5 ml. of *ethanol* and 15 ml. of *naphtha,* until no more color is extracted.

Color Measurement. Employ the Peterson and Hughes procedure [26] shortened as noted above.

NOTE. For sweet potatoes, boil 30 minutes with 50 ml. of *ethanol* instead of 12% ethanolic potassium hydroxide solution.

Sircar Naphtha-Acetone-Alkali Photometric Method.[27] The common Indian green vegetables were analyzed by this method.

APPARATUS. *Hilger-Sector Photometer.*

PROCESS. *Extraction.* Wash and slice the green material, dry on trays at 45 to 50°, and grind to pass a 60-mesh sieve. Digest 2 to 5 g. of the ground material in 200 ml. of *naphtha* for 24 hours. Filter, wash with *naphtha,* followed by *85% acetone* until no more color is removed.

Saponification. Wash the combined extracts with water to remove the acetone, then digest with *29% ethanolic potassium hydroxide solution* for 12 hours with occasional stirring and wash with water to remove chlorophyllins. If some of the green coloration persists, repeat the saponification and washing. Dry the naphtha solution over *anhydrous sodium sulfate*, concentrate *in vacuo*, then dilute to a suitable volume.

Color Absorption. Determine in the Hilger instrument, using wave length 4900 Å.

CALCULATION. Compare with a curve plotted from results on pure carotene.

EXAMPLES. Lettuce 21, cabbage (white leaves) 8.3, and cabbage (green leaves) 25 γ/g.

Moore Dicalcium Phosphate Colorimetric Method.[28] The method was devised at the Michigan State College to overcome the interference of chromogens developed during the curing of hay and silage. It is doubtless applicable to leaf vegetables.

APPARATUS. *Tswett Extractor.* Blow out the bottom of a test tube 19 cm. long and 2.2 cm. in diameter and attach a short piece of glass tubing.

Colorimeter.

PROCESS. *Ethanol Extraction.* Grind 2 to 4 g. of the dried sample with *sand* in a mortar, transfer to a 250-ml. Erlenmeyer flask, and add 50 ml. of *aldehyde-free ethanol.* In the case of fresh material, grind with the *ethanol* before transferring to the Erlenmeyer flask. Reflux for 30 minutes on a water bath, cool, and decant the ethanol extract into a 500-ml. separatory funnel. Add to the residue 25 ml. of *ethanol* and repeat the extraction and decantation.

Naphtha Extraction. Add to the residue *naphtha* (about 50 ml.) together with sufficient *ethanol* (about 25 ml.) for complete dispersion (total 100 ml.), and extract as before.

Dilute the total extract with water to an ethanol concentration of 80%, shake, and remove the aqueous layer. Shake the aqueous layer with three more 30-ml. portions of *naphtha;* this suffices for the removal of all the carotene, although some of the extraneous color remains in the ethanol.

Ethanol Removal. Wash the naphtha extract six or seven times with 100-ml. portions of tap water for the complete removal of the ethanol, transfer to an Erlenmeyer flask, and evaporate to 20 to 25 ml. in a hot water bath with suction.

Foreign Pigments Removal. Plug the delivery tube of the Tswett extractor with a small wad of cotton, half fill with *dicalcium phosphate*, pack lightly by suction and tamping, add a small amount of *anhydrous sodium sulfate*, and cover with a large wad of cotton. Run 30 ml. of the *naphtha* through the column, then the concentrated extract of the sample, and finally 60 ml. of *naphtha.* The various pigments are absorbed in bands, except carotene which passes through in solution. Concentrate if necessary and make to volume.

Color Reading. Determine the carotene colorimetrically either by direct comparison with a *standard solution* or by the use of a photoelectric colorimeter.

Moore and Ely Ethanol-Naphtha Food Blender Colorimetric Method.[29] The method here described, representing work done at the Michigan Agricultural Experiment Station, employs a food blender in conjunction with a foaming solvent. Coleman and Christie [30] employ a high speed stirrer for extracting carotenoids from flour; Davis [31] and workers at Purdue University use a blender for cutting and extracting samples.

Apparatus. *Waring Food Blender* (Waring Corporation, New York, N. Y.).

Photoelectric Colorimeter.

Reagent. *Foaming Solvent.* Mix 100 ml. of ethanol and 75 ml. of naphtha for each analysis.

Process. *A. Fresh Material.* Weigh 1 to 4 g. of the chopped sample into the blender, add the *foaming solvent*, and run the blender for 5 minutes, adding more *ethanol* if necessary to produce a foam. Allow to settle, transfer the supernatant liquid to a separatory funnel, dilute to 80% ethanolic concentration, and draw off the lower layer. Wash the residue with 30 ml. of *naphtha* and use this naphtha to extract the ethanol layer in the separatory funnel. Repeat the treatment of the residue and the ethanol layer twice with 30 ml. of *naphtha* and wash the combined naphtha extracts with tap water six or seven times to remove all ethanol. Concentrate under vacuum in an Erlenmeyer flask to about 25 ml., heating by means of running hot water. Remove foreign pigments by passing the extract through a column of *dicalcium phosphate.* Make the eluate up to a definite volume and determine the carotene in a photoelectric colorimeter.

B. Dry Material. Wash 2 g. of the sample on a filter paper with 25 ml. of tap water at 60 to 70°. Draw off the excess of water by light suction and treat the moist material as above.

Lease and Mitchell Ethanol Extraction Photoelectric Method.[32] Most of the methods include directions for saponification with *potassium hydroxide in ethanol or methanol* as the first step in extracting carotene. Lease and Mitchell (South Carolina Agricultural Experiment Station), however, found that procedure inapplicable to certain cooked vegetables and some raw products, owing to apparent polymerization of carbohydrate by the alkali with the formation of a resinous film that reduces the amount of carotene extracted. From such products, *ethanol* extracts the full amount of the carotene.

Examples. The following photoelectric results (checked colorimetrically) by extraction with ethanol and by potassium hydroxide in ethanol respectively are reported: sweet potatoes, raw 130 and 124, boiled 130

and 5, baked 130 and 2, baked 130 and 130 (subsequent to refluxing with alcoholic potassium hydroxide and water), stored four months (5°) 127 and 83, (37°) 124 and 25; cooked carrots 302.5 and 100.9; summer squash, raw 15 and 4, cooked 13 and 4; pumpkin (canned) 92.6 and 77.2; hay of various grasses and legumes 32 to 41 and 32 to 40.1 γ/g.

β-Carotene

Buxton and Dombrow Heptane Spectrophotometric Method for Alfalfa Meal.[33] See also the Buxton Method for determination of α- and β-carotene in yellow corn. Although devised for alfalfa, the method may be used for leaf vegetables.

Apparatus. Modified Bausch & Lomb *visual spectrophotometer* equipped with a *Duboscq colorimeter* and a rotating sector.

Process. *Saponification and Extraction.* Reflux on a steam bath for 30 minutes 5 g., or other suitable quantity, of the dehydrated sample, in a 250-ml. flask with 75 ml. of *10% potassium hydroxide in ethanol.* Cool, add 100 ml. of purified technical *heptane*, shake thoroughly, allow to settle, and decant the supernatant liquid into a 500-ml. separatory funnel. Reextract the residue with 50-ml. portions of *heptane* three times or until the solution is colorless.

Purification. Wash the combined heptane extracts free from chlorophylins, flavones, xanthophyls, and alkali by shaking vigorously with about five portions of *90% methanol*, testing with phenolphthalein, then reextract the first methanol portion with 50 ml. of *heptane*. Distil the heptane fraction to small volume in an atmosphere of nitrogen with the aid of suction.

Spectrophotometric Reading. Take up the residue, consisting chiefly of carotene, in *heptane*, transfer to a 50-ml. volumetric flask, and make up to the mark with *heptane.* Make several readings in a visual spectrophotometer at 4500 Å and record the average.

Calculation. The extinction coefficient ($E^{1\%}_{1\,cm.}$ 4500 Å) of pure β-carotene in the heptane solution, as determined by Buxton and Dombrow, is 2380. Use this value for the extinction coefficient as a factor (F) in the following equation:

$$G = \frac{S \times F}{R \times C}$$

in which G is gammas of carotene for a 1% solution, S is the screen factor, R is the reading in centimeters, and C is the concentration.

Carotene and Lycopene

See also Part I, C10.

Fraps, Kemmerer, and Greenberg Magnesium Carbonate Adsorption Method.[34] It is well known that tomatoes, red peppers, and some other fruits and vegetables contain lycopene in addition to the more common carotenoids.

Reagents. *Activated Magnesium Carbonate (Lycopene Reagent).* Heat 100 g. of $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$ in an electric furnace at 200° for 1 hour. Make tests to insure that the reagent does not adsorb carotene from a solution (0.5 to 0.8 γ/g.) and that 3 to 5 g. adsorb all the red pigment from 50 ml. of lycopene solution (0.5 to 0.8 γ/g.). If carotene is adsorbed, add 3 ml. of water to the $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$ and test, repeating the addition if necessary, then test again with lycopene solution. Keep the reagent in a tightly closed jar and test before use.

Process. As in the Fraps and Kemmerer Magnesium Hydroxide Adsorption Method for carotene above.

Examples. The crude carotene in the sample and the pure carotene after treatment with xanthophyl reagent and with lycopene reagent respectively were as follows: old alfalfa hay 19.2, 15.0, and 14.8,

watermelon 46.4, 42.6, and 10.8, dried apricots 45.6, 41.7, and 31.5, red peppers 4.7, 2.9, and 2.1, and tomatoes 43.9, 43.8, and 18.0 γ/g.

Riboflavin

Conner and Straub Decalso-Supersorb Fluorometric Method. See Part I, C10.

Mackinney and Sugihara Modification.[35] Process. The extraction and preparation of the sample are carried out as directed by Conner and Straub, except that in the case of fruits it was found necessary to add 10 ml. of *pectinol* (1 g. in 25 ml.) to 50 ml. of the extract, in addition to the *clarase*. After incubating at 45° for 2 hours, a 10- to 20-ml. aliquot is heated to boiling with 5 ml. of *2% acetic acid*. The solution is diluted to 50 ml. with the *buffer* and a 15-ml. aliquot is treated for at least 3 minutes with 1 ml. of *potassium permanganate solution* and decolorized with 3 ml. of *3% hydrogen peroxide*. The solution is filtered and compared with buffered standards at pH 6 in a Coleman fluorophotometer.

Examples. Asparagus, fresh 2.19, blanched 2.60; broccoli, fresh 0.77, dehydrated 3.71 to 7.25; peas, fresh 0.91 to 1.16, cooked 1.00, dehydrated 4.16 to 4.38; spinach, dehydrated 10.2; and grass, dehydrated 5.28 γ/g.

Ascorbic Acid

See Part I, C10, and Part II, C1, D2, and G1.

Mindlin and Butler Method. See Part I, C10.

Morell Photometric Modification.[36] The features of this modification are the method of extraction, centrifuging instead of filtering, and changes in the reagents to cover a wider range (1 to 14 γ). As carried out at the U. S. Department of Agriculture, Bureau of Plant Industry, an analyst with two assistants readily can run 120 samples daily in connection with breeding experiments.

Apparatus. *Evelyn Photoelectric Colorimeter*, with green filter No. 520 (transmission limits 495 to 550 mμ).

Absorption Test Tube, 17.5 x 2.2 cm., agreeing within 0.25 galvanometer unit.

Blender.

Reagents. *m-Phosphoric Acid Solution*, 3%.

Sodium Citrate Buffer. Dissolve 211 g. of $H_3C_6H_5O_7 \cdot H_2O$ in 2 liters of *1.0 N* NaOH solution.

Buffer, at pH 3.6 ($\pm$0.1). A mixture of 3200 and 868 ml. respectively of the two foregoing solutions.

Indophenol Solution. Dissolve 34.4 mg. of 2,6-dichlorophenol indophenol (Eastman Kodak Co.) in 1 liter of water. Store all solutions in the refrigerator overnight and use within 5 days.

Ascorbic Acid. Tested by titration with standard iodine solution.[37]

Standard Curve. Prepare a fresh solution of 25 mg. of *ascorbic acid* in 250 ml. of *buffer at pH 3.6*. Add 1- to 14-ml. portions, by 1-ml. increment, to 100-ml. volumetric flasks and dilute to the mark with the *buffer at pH 3.6*. Add 5-ml. portions of the *indophenol solution* from the same pipet to each of a series of colorimeter test tubes. Set the colorimeter in triplicate at 100% transmission, using a tube containing 5 ml. of *buffer at pH 3.6*, 5 ml. of the *indophenol solution*, and a few crystals of *ascorbic acid* for complete decoloration. Record and hold constant the center setting, with no tube in the instrument. Deliver quickly 5 ml. of a sample solution from an Ostwald pipet into 5 ml. of the indophenol solution, and shake vigorously for 5 seconds, then take a reading 15 seconds after initial mixing and again at 30 seconds. After observing the values for all 14 samples, obtain a blank reading in triplicate, using 5 ml. of the *buffer*. The difference between the readings at 15 and 30 seconds should not exceed 0.5 galvanometer unit.

By the method of least squares, the equation for the line best satisfying the experi-

mental points is

$$Y = 0.0441X \times 0.0137$$

in which X is gammas of ascorbic acid per milliliter and Y is the log of the galvanometer readings for samples minus that for the blank.

PROCESS (BEANS AND CABBAGE). *Extraction.* Place in the container of the blender 100 ml. of *3% m-phosphoric acid solution*, 25 g. of fresh tissue, and mix at high speed for 2 minutes. Filter about half of the pulpy suspension through a dry pleated No. 12 Whatman paper, discard the first 10 ml., and collect 10 ml. of the clear filtrate in a dry Erlenmeyer flask. Transfer an aliquot (3 ml. for cabbage, 5 ml. for string beans) to a 50-ml. volumetric flask, add *sodium citrate buffer* to bring the *p*H to about 3.6 (0.25 ml. per milliliter of aliquot for cabbage, string beans, and several other vegetables), then make up to volume with *citrate-phosphate buffer*. The final *p*H should be 3.6 ± 1.

Reading. See Standard Curve above.

METALS

See Part I, C8*b*.

2. CANNED VEGETABLES

ALCOHOL-INSOLUBLE MATTER IN CANNED PEAS

Official A.O.A.C. Method.[38] The official method depends on the difference in the amount of matter soluble in 80% ethanol by volume.

PROCESS. *Liquid Removal.* Drain the contents of the can, spread evenly on an 8-mesh-per-inch (2.5-cm.) screen (8 inches for containers of less than 3 pounds, 12 inches for larger containers), then add a volume of water double the original volume of the sample. Pour the peas back on the screen, spread evenly, tilt without shifting the peas, and drain for 2 minutes. Wipe the moisture from the lower surface of the screen and grind the peas in a food chopper until the cotyledons are reduced to a homogeneous paste.

Ethanol Treatment. To 20 g. of the pulped material, in a 600-ml. beaker, add 300 ml. of *80% (by volume) ethanol*, stir, cover, bring to a boil, and boil gently for 30 minutes. Fit into a Büchner funnel a filter paper previously dried for 2 hours in a water oven, cooled in a covered dish in a desiccator, and weighed. Apply suction and collect the insoluble matter on the paper without running over the edges. Suck dry, wash with the *80% ethanol* until the washings run through clear and colorless. Transfer the paper and insoluble solids to the dish, dry, cool, and weigh as before.

CALCULATION. Obtain the percentage of ethanol-insoluble solids.

FIELD CORN IN CANNED SWEET CORN

A.O.A.C. Microscopic Method.[39] The tentative A.O.A.C. method depends on the presence of dextrins in sweet corn that form a brown color with *iodine in potassium iodide solution*, obscuring the blue color of the starch grains and their absence in field corn.[40] Sections are cut and examined under the microscope. The description of the procedure urges caution in drawing conclusions since immature kernels of sweet corn do not contain enough dextrin to produce the color; it does, however, give the technique of separating the fragments unquestionably derived from field corn, weighing, and calculation.

3. TOMATO PRODUCTS

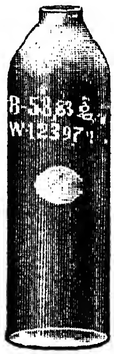
The methods are designed for catsup and similar products.

SPECIFIC GRAVITY

National Canners Association Method.[41] APPARATUS. *National Canners Association*

Specific Gravity Bottle (Fig. 126). In calibrating, fill with water, strike off with a straightedge, wipe clean, and weigh at 20°.

PROCESS. Cool the sample to 16 or 18°, fill the flask with the pulp, and centrifuge at 1000 r.p.m. with a counterpoise in the opposite cup. Add pulp to the top and whirl again. Insert the thermometer without introducing air; when the temperature reaches 20° remove the thermometer, fill with more pulp at 20°, strike off, clean the outside of the flask, and weigh to nearest 0.01 g.



Courtesy of Methods of Analysis, A.O.A.C. 1935, p. 499

FIG. 126. National Canners Association Specific Gravity Bottle.

CALCULATION. Divide the weight of the pulp by the weight of water at 20°, thus obtaining the specific gravity at 20°/20°.

WATER

(Moisture)

See also Part I, C2, and Part II, D2.

Leonhard Xylene Distillation Method.[42] For the determination of moisture in tomato products, Leonhard prefers distilling with xylene and measuring the volume of the distillate to drying in an oven and other methods. Doubtless his procedure also may be employed for fruit products and, on the other hand, methods described under fruit products are suited for tomato products, in both cases with suitable adjustment of quantities of the sample, time of heating, and minor details.

APPARATUS. *Special Distillation Assembly*, consisting essentially of a 300-ml. Erlenmeyer flask, heated in a sand bath, and a two-mouthed receiver provided with a closed stem, cooled in an inverted bottle from which the bottom has been removed. One of the two mouths of the receiver carries a small tube drawn out to an opening 2 mm. in diameter. The graduations on the stem are for measuring the moisture, those on the main portion for observing the progress of the distillation.

PROCESS. Weigh 5 to 10 g. of the sample into an aluminum boat and introduce into the Erlenmeyer flask together with 150 ml. of *xylene*. Place 1 ml. of *1 + 1 potassium hydroxide* in the receiver and distil at the rate of 150 ml. of distillate in 20 minutes. While shaking gently the receiver, drive into it any moisture deposited in the delivery tube by heating with a small flame.

Reading. Cool for 2 hours, measure the aqueous column, and make a deduction for 1 ml. of the alkali solution added at the start.

TOTAL SOLIDS

Tentative A.O.A.C. Vacuum 70° Method for Canned Products Only. Weigh into a flat-bottom dish a charge containing between 9 and 12 mg. per sq. cm. of drying surface. Distribute evenly over the bottom, adding water if necessary. Evaporate rapidly at 70° in a vacuum oven with release cock partly open so that the vacuum does not exceed 450 mm. of mercury. Admit through the release cock air dried by bubbling through sulfuric acid. Examine at hour and half-hour intervals thereafter until apparently dry, then nearly close the release cock so as to admit 2 bubbles of air per second and dry at 70° for 4 hours at a pressure not exceeding 100 mm.

Insoluble Solids

Tentative A.O.A.C. Method. Wash 20 g. of the sample four or five times with hot water in a centrifuge bottle, whirling after each addition, and decant on a Büchner funnel through one of two filter papers, thoroughly dried for 2 hours at 100° and weighed. Use the second paper if the first becomes clogged. Wash four or five times, transfer the solid matter to the filter, dry in a covered dish at 100° for 2 hours, cool in a desiccator, and weigh.

Total Acids; Volatile Acids; Non-Volatile Acids

The chief acid of the tomato is citric; the acid added in preparing catsup and similar products is acetic in the form of vinegar. The following are tentative A.O.A.C. methods.

A. Total Acids. Dilute 5 g. of the sample with water and titrate with *standard 0.1 N sodium or potassium hydroxide solution* in the usual manner; 1 ml. of 0.1 N alkali = 0.0064 g. of anhydrous citric acid.

B. Volatile Acids. Determine the volatile acids in the distillate obtained by the steam distillation of 25 g. of the sample as directed in Part II, F1; 1 ml. of 0.1 N alkali = 0.0060 g. of acetic acid.

C. Fixed Acids. Multiply the percentage of volatile acids by 1.067 and subtract from the percentage of total acids, thus obtaining the percentage of fixed acids in terms of citric acid.

Lactic Acid

Hillig Ferric Chloride Colorimetric Method.[43] Apparatus and Reagents. As given for the Hillig Method, Part II, G1.

Process. Weigh 20 g. of tomato paste, catsup, or chili sauce (or 40 ml. of tomato juice) into a small beaker, transfer to a 200-ml. volumetric flask with water, make up to the mark, shake, and filter. If the filtration is slow, centrifuge, decant, add a small amount of Filter-Cel, and shake. Transfer 50 ml. of the filtrate to a continuous extractor, add 1 ml. of *1 + 1 sulfuric acid*, and proceed with the determination as described in Part II, G1, with the following exceptions. First, extract for 3 hours instead of 2 catsup or other product containing acetic acid. After the extraction evaporate the ether, make up to 50 ml., and remove the acetic acid by steam distillation until the distillate measures 250 ml. Evaporate to 20 ml., neutralize with *saturated barium hydroxide solution*, transfer to a 110-ml. volumetric flask with *ethanol*, and then proceed as in the method for Milk (Part II, G1), reading the color value in the photometer equipped with light filter 460 mμ instead of 450 mμ.

Calculation. Obtain the percentage of lactic acid (P) by the formula

$$P = \frac{50}{250} \times \frac{50}{110} \times \frac{100}{55} \times A = 0.1653A$$

in which A is the milliliters of aliquot taken in the final analysis.

Examples. Lactic acid in tomato juice (13 samples) 3.3 to 8.3, catsup (6 samples) 23.6 to 29.0, paste (2 samples) 19.8 to 25.4, pulp (1 sample) 5.5, and chili sauce (2 samples) 21.2 to 27.2 mg. per 100 g.

Sugars

See Part I, C6*a*.

Sodium Chloride

A.O.A.C. Official Method. See Part I, C8*a*. Calculate as sodium chloride.

Beacham Tentative Rapid Method.[44] Process. *Silver Chloride Precipitation.* Weigh 5 g. of the sample into a 100-ml. volumetric flask and add *80% ethanol* to about 50 ml. Shake well, add 1 ml. of *nitric acid* and an accurately measured excess of *standard 0.1 N silver nitrate solution*. Dilute to the

mark with *ethanol*, transfer to a centrifuge bottle, and whirl at about 1800 r.p.m. for 5 minutes. Pipet 50 ml. of the supernatant liquid into a 300-ml. Erlenmeyer flask and add 2 ml. each of *saturated ferric ammonium sulfate solution* and *nitric acid*.

Titration. Add *standard 0.1 N ammonium thiocyanate solution* to a permanent light brown color.

CALCULATION. Obtain the percentage of salt (S) by the formula

$$S = \frac{(0.5N - T) \times 0.005843 \times 100}{2.5}$$

in which N and T are the number of milliliters of silver nitrate and thiocyanate solutions respectively.

ASH; ALKALINITY OF ASH; METALS; PRESERVATIVES

See Part I, C2*f*, C8*b*, and C13.

SAND

A.O.A.C. Tentative Direct Method.[45] The usual method of ashing and determination of the ash insoluble in hydrochloric acid is not applicable owing to the uneven distribution of the sand and the small sample.

PROCESS. Weigh 100 g. of the very thoroughly mixed sample into a 2- to 3-liter beaker nearly filled with water and stir well. After 5 minutes, decant the supernatant liquid into a second beaker. Refill the first beaker with water, mix, and after 5 minutes decant from the second into a third beaker and from the first into the second. Refill and mix the contents of the first beaker, repeating the operations, decanting from the third beaker into the sink, until the light material is washed from the sample. Collect the sand from the three beakers on a weighed Gooch crucible, dry, ignite, and weigh.

MOLDS, YEASTS, SPORES, AND BACTERIA

Howard Counting Method.[46] APPARATUS. *Compound Microscope*, with ocular and objective combinations magnifying 90, 180, and 500 diameters, adjusted so as to be parfocal. Lower power field area 1.5 sq. mm. (circle with 1.382 mm. diameter); high power working distance sufficient for use of blood-counting cell.

Ocular Drop-In Cross-Ruled Disk, cross-ruled in sixths of the ocular diaphragm.

Blood-Counting Cell. Ruled in the Thoma or the *old* Neubauer system.

Howard Mold-Counting Cell. Like a blood-counting cell, but with unruled disk about 19 mm. in diameter.

PROCESS. *Molds*. Add *mold-free gum* to thin products and water to thick products to 8.37 to 9.37% of solids, taking account of salt or other substances materially increasing the solids content.

Clean the Howard cell so as to produce Newton rings between the slide and cover-glass. Remove the cover and place a small drop of the *well mixed* sample on the central disk. *Spread evenly* with a knife blade and cover so as to give an even layer. Avoid a drop more than sufficient to fill the space between the center disk and the cover-glass.

Examine fields of 1.5 sq. mm., adjusting the draw tube so that the field is 1.382 mm. in diameter. If necessary, have a mechanic make a drop-in ocular diaphragm with an accurately cut aperture. Determine the area of the view field by means of a stage micrometer or the ruling on the blood-counting cell with cognizance that the diagonal of a square with 0.977-mm. sides is 1.382 mm. For use adjust the blood-counting cell so that the circle of the field of view cuts off the necessary amount from each corner of the ruled square. With proper adjustment, 0.15 cm (0.00015 ml.) of liquid is examined.

In two or more mounts, examine at least 25

fields representative of all sections. Record as positive (+) only when the aggregate length of not more than 3 filaments exceeds one-sixth the diameter of the field. Report the percentage of all the fields examined containing mold filaments.

Yeasts and Spores. Fill a 50-ml. graduated cylinder with water to the 20-ml. mark and add the sample to the 30-ml. mark. Shake vigorously 15 or 20 seconds. Use a 100-ml. cylinder, 80 ml. of water, and 10 ml. or g. of tomato sauce, paste, or other product of heavy consistency and of a product rich in organisms. Dilute still further exceptionally thick or dry pastes. Pour the mixture into a beaker, stir well with a scalpel, let stand 3 to 5 seconds, and remove a small drop to the central disk of the well-cleaned blood-counting cell. Cover immediately and make the count after standing at least 10 minutes, using a magnification of 180 to 220 diameters.

Count the number of yeasts and mold spores (avoiding counting twice those on the lines) on half the ruled squares on the disk, that is, the number in 8 of the blocks, each with 25 small ruled squares. The total number is equivalent to 1/60 cmm. (1/60,000 ml. of the original product) of a 1 + 2 dilution. Multiply by 3 for a 1 + 8 dilution.

Bacteria. Make the count on the mount used for yeasts and spores, but allow to stand not less than 15 minutes before examination. With a magnification of 500 diameters, count the number of rod-shaped bacteria having a length 1.5 times their width in 5 areas (1 near each corner and 1 near the center of the ruled portion), each including 5 of the small squares. Avoid overlooking rod-shaped forms that during motion may momentarily appear isodiametric. Multiply the total number by 480,000 or 1,440,000, thus obtaining the number per milliliter for a 1 + 2 or 1 + 8 dilution respectively.

Micrococci. Counting of micrococci is regarded at present impracticable.

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D. FRUIT FOODS

In the botanical sense a fruit is the ripened pericarp or fruit coat of the ovary together with the enclosed seeds. The fruit flesh may be dry or succulent, thick or thin, edible or inedible. In the pomological and commercial sense the term is restricted to the succulent development of one or more of the floral parts which is usually the pericarp, but sometimes it is layers of the seed coat (pomegranate), the aril or seed appendage (litchi and longan), or even the fruit stem (cashew apple). The petiole of rhubarb is similar in character to the flesh of fruits and is used for a similar purpose. Both fruit and fruit products are treated in this chapter.

Microscopic Structure (Figs. 127 to 132). Although most temperate fruits belong in the rose family, their structure is highly varied, particularly as to the epicarp, often with hairs, and the seed coats of the starch-free seeds, thus permitting to some extent the identification of the source of jams and preserves. Strawberry, raspberry, blackberry, and other small-fruit jams may be readily distinguished by the gross and minute structure of the so-called seeds which are radically different in structure from those of currants and gooseberries. Pomes, in the green stage, contain starch in the fruit flesh which disappears on ripening. The same is true of the starch so abundantly present in green bananas as to furnish a source for commercial starch. The needle-shaped crystals of the pineapple are characteristic. The fruit flesh of citrus fruits is a disappointment to the microscopist who, however, is consoled by the characteristic tissues of the seeds which permit identification. At least fifty tropical fruits, unknown to the northern palate, which the writers have traveled far to study, show endless variety in cell structure.

The technique for the examination of jams, preserves, mincemeat, and pie fillings consists in the separation of the seeds, skin, cores, and stone cell aggregations from the pulp tissues and the examination of each separately. Such gross frauds as the addition of grass seeds to artificial jams, once prevalent, are no longer practiced in the United States. The substitution of the pulp of cheaper fruits (e.g. apple and plum) is difficult even for the expert to detect.

LEGENDS OF ILLUSTRATIONS ON THE FACING PAGE

Only six of the most common fruits are here illustrated. The magnification is ×160.

FIG. 127. Apple. Elements in surface view. Fruit coat: *epi* epicarp, *hy* hypoderm, *st* stone cell, *p* parenchyma with rosette crystal and small starch grains, *v* vessels and *f* fiber of fibrovascular bundle, *end* fibers and crystal cells of endocarp, and *t* hairs of suture.

In pared and cored apple products, only occasional fragments of the epiderm, hypoderm, and core are present. Crystal rosettes, also in the unripe fruit starch grains, occur in the fruit flesh.

Stone cells are numerous in the pear and still more numerous in the quince.

FIG. 128. Orange. Elements in surface view. Fruit coat: *mes* mesocarp elements showing *ol* oil drops, *cr* oxalate crystals, *h* hesperidin crystals, and *p* spongy parenchyma. *ves* vesicle elements showing oxalate crystals and groups of small chromatophores.

Oil drops in special cavities and oxalate and hesperidin crystals in thick-walled cells occur in the rind; oxalate crystals and orange chromatophores characterize the cells of the vesicles.

FIG. 129. Banana. Elements in surface view. Fruit coat: *epi* epicarp with stoma, *hy* hypoderm with raphides, *p* starch parenchyma, *ol* oleoresin cell with oil drops, *fv* fibrovascular bundle elements, and *end* endocarp. *S* seed-coat elements. *N* perisperm elements.

Large starch grains with eccentric hilum are numerous in the green fruit, but disappear on ripening. Oleoresin cells are abundant in the peel. Groups of raphides occur here and there in the hypoderm.

FIG. 130. Pineapple. Elements in surface view. Bract: *B* outer epiderm with scale-like hairs, stoma, and stegmata, and inner epiderm with stegmata. Perianth tube: *aep* outer epiderm with stoma and stegmata, hy^1 hypoderm, and *iep* inner epiderm with stegmata. Fruit coat: *epi* epicarp, hy^2 hypoderm, *mes* mesocarp cells with raphides, *v* vessel, *f* fiber, *scl* sclerenchyma layer, and *end* endocarp.

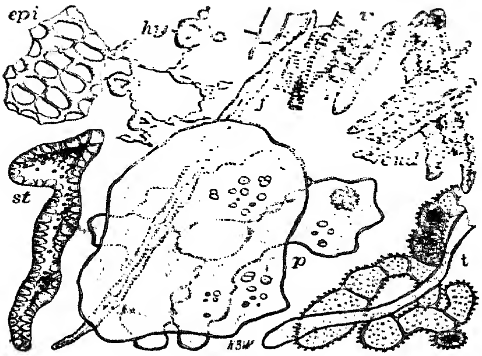
The striking elements of the inedible outer portion of the fruit are removed in paring. Raphides are conspicuous in the edible portion.

FIG. 131. American Grape. Elements in surface view. Fruit coat: *epi* epicarp, mes^1 mesocarp cells with raphides, *fv* fibrovascular bundle elements, and *end* endocarp; mes^2 mesocarp with sugar crystal formed in alcoholic specimen. Seed coat: *aep* outer epiderm, *hy* hypoderm with raphides, *st* stone cell layer, *l* lattice cells, and *iep* inner epiderm. *E* endosperm with aleurone grains.

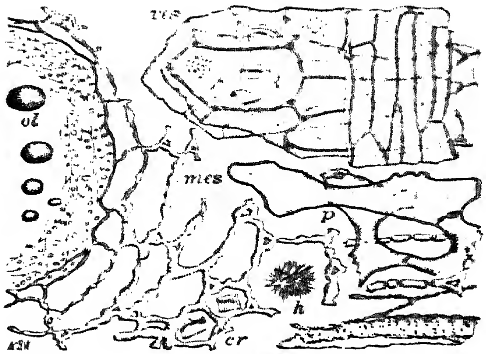
The beaded epicarp is much like that of certain other fruits. Raphides occur in the parenchyma of the fruit flesh and oxalate rosette crystals in the bundles of the veins.

FIG. 132. Strawberry. Elements in surface view. Fruit coat: *epi* epicarp with hair and stoma, and *hy* hypoderm with rosette crystals. *St* style elements. *S* seed-coat elements.

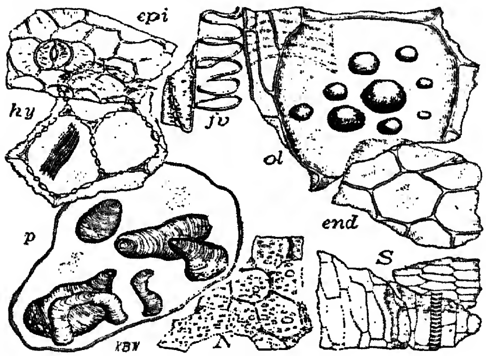
The epiderm of the fruit with an occasional pointed hair distinguishes the strawberry from the red raspberry with a tangle of interlocking crooked hairs and from the black raspberry and the wineberry without hairs. The lens characters distinguish the seeds and styles of the four fruits, all being learned by observation of water mounts.



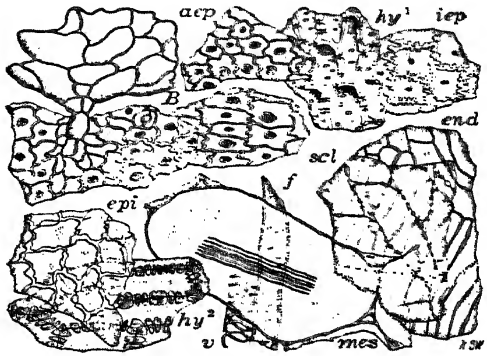
Apple



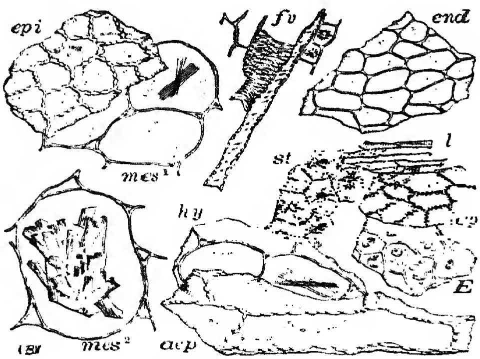
Orange



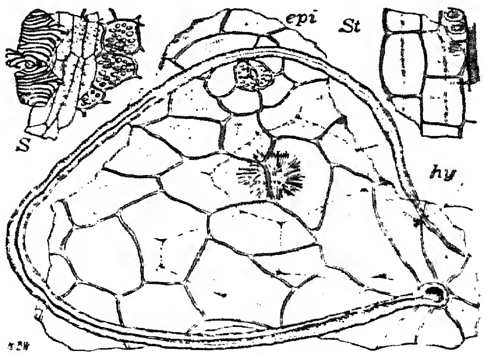
Banana



Pineapple



American Grape



Strawberry

Figs. 127-132.

PART II, D1 FRUIT

Average Composition of the Edible Flesh of Fruits

	Solids, Total	Solids, Insol.	Protein	Acid	Invert Sugar	Sucrose	Ash, Total	Ash, Alk.*
	%	%	%	%	%	%	%	ml.
Apple	16.43	....	....	0.61 †	7.92	3.99	0.27	..
Apricot	10.47	1.79	....	1.55 †	5.01	1.42	0.72	76
Banana	27.54	3.40	1.16	0.41 †	11.69	10.37	0.86	91
Blackberry	12.73	5.45	0.92	0.91 ‡	4.67	0.16	0.59	69
Blueberry	15.13	....	0.50	0.93 ‡	4.89	....	0.11	..
Carob §	88.50	....	4.50	....	11.24	23.17	2.72	..
Cherry, sweet	12.43	1.73	....	0.73 †	9.60	....	0.59	58
Cranberry	11.9	....	0.31	2.61 ‡	3.7		....	..
Currant, red	12.97	6.90	1.37	2.21 ‡	3.44	0.00	0.60	63
Date §	84.60	....	2.1	....	....	....	1.3	..
Fig	14.98	2.37	1.40	0.21 ‡	10.80		0.49	..
Gooseberry	12.00	3.98	....	1.95 ‡	4.87	0.18	0.43	33
Grape, American	22.60	....	1.3	....	....	....	0.5	..
Grapefruit	9.94	2.70	0.58	0.77 ‡	2.60	4.77	0.46	..
Guava	21.32	12.94	0.88	0.77 ‡	4.49	0.45	0.69	77
Lemon skin ‖	23.62	....	1.79	....	....	....	0.52	..
" juice	9.21	....	0.32	5.86 ‡	0.87		0.20	..
Mango	16.76	1.76	0.43	0.39 ‡	3.56	10.06	0.47	47
Mangosteen	19.8	1.9	0.50	0.49 ‡	4.20	12.63	0.23	29
Orange	11.38	2.70	0.72	2.65 ‡	4.45	5.29	0.57	..
Peach	....	....	0.35	0.77 †	1.98	5.70	0.50	..
Pear	12.46	....	....	0.56	9.15	....	0.37	..
Persimmon	21.54	....	0.57	0.14 †	15.51	....	0.45	..
Pineapple	14.17	1.52	0.42	0.86 ‡	3.91	7.59	0.40	53
Plum	22.8	2.9	....	1.7 †	13.7	4.8	0.60	..
Pomegranate	21.73	....	1.33	0.37 ‡	11.61	1.04	0.76	..
Prickly pear	9.79	....	1.54	....	5.60		0.33	..
Quince	19.0	7.1	....	1.0 †	8.2	0.16	0.73	..
Raspberry, red	13.28	6.87	0.98	1.58 ‡	3.52	0.80	0.54	60
Soursop	19.03	5.45	1.65	0.70 †	13.07	0.00	0.41	53
Strawberry	8.74	3.39	0.59	1.31 ‡	3.22	0.46	0.62	63
Tamarind §	69.51	17.68	3.43	17.33 ¶	18.74	2.58	1.82	..

* Ml. 0.1 N acid per 100 g. fruit. † Calculated as malic. ‡ Calculated as citric. § Cured
‖ Volatile oil 1.01%. ¶ Calculated as tartaric.

Chemical Composition. So far as the flesh is concerned, fruits as a class serve chiefly as a source of sugars, organic acids, vitamins, and flavors. Nitrogenous substances and fat occur in considerable amount only in the seeds which either are not eaten or, as in berries, pass through the digestive system with little change. The avocado and the olive (classed both as vegetables and oil fruits) are notable exceptions. Starch occurs in unripe bananas and in smaller amount in green apples, but during ripening changes to sugars. Acids also diminish with corresponding increase of sugars.

The average composition of the edible flesh of fruits given in the accompanying table, although based on meager data, particularly as regards acids, will serve for general comparison.

1. FRUIT; DRIED FRUIT

Analytical Methods. The methods differ from those given for candied fruits, sirups, jellies, jams, and preserves merely in that the absence of added sugar makes it expedient to employ somewhat different quantities of the sample for the determinations, namely, more for sugars and less for most of the other constituents.

Sample

Separate fruits and dried fruits so far as possible into edible and inedible portions and determine their percentage. Reduce the edible portion if possible to a homogeneous pulp. Use portions of considerable amount for the several determinations, thus reducing to a minimum any lack of uniformity, and make determinations, such as of sugar and acid, on suitable aliquots. If a homogeneous mixture of the whole edible portion is not obtainable, separate into juice and marc or pomace, determine the percentage of each, and analyze separately. Dried fruits differ from fresh fruits chiefly in that a portion of the water has been evaporated and they are treated accordingly. Treatment of dried fruits with sulfur fumes serves partly to improve the appearance and partly to prevent spoilage.

Solids

(Moisture)

Official A.O.A.C. Covered Dish 70° Vacuum Method. For fresh fruits employ 20 g. and for dried fruits only 5 to 10 g., of the pulped sample in a tared covered metal dish 8.5 cm. in diameter and dry at 70° for 6 hours under a pressure not exceeding 100 mm. of mercury in a current of air (about two bubbles per second) dried by passing through sulfuric acid. Ignore any drop in temperature during the initial stages. Replace the cover, cool in a desiccator, and weigh. Use only 5 g. of highly saccharine fruits such as raisins, mix with 2 g. of asbestos or sand, and evaporate on the steam bath before drying in the vacuum oven.

Pritzker and Jungkunz Tetrachloroethane Distillation Method for Dried Fruits.[1] Although tetrachloroethane is specified, xylene was found to give equally good results.

Apparatus. *The Distillation Assembly* (not shown), consisting of two ingenious special parts and an Erlenmeyer flask, has a number of novel features. It doubtless can be employed in the determination of moisture in other food products. One of the special parts consists of a vertical condensing chamber (*a*) extended below as a narrow graduated tube of 15 ml. capacity and joined at the side to a vertical tube (*d*) connected with the distilling flask (*f*). A double-bent small-bore tube (*e*) connects by a stopcock (*b*) the bottom of the graduated tube with tube *d* at the level of the 10-ml. graduation mark. The other special part is a Normann (outer surface) condenser.

The condenser is readily inserted and its proper position is assured by the collar that

fits closely to the top of *a* and the prongs that prevent contact with the walls and direct the condensed liquid into the graduated portion of *a*. The 250-ml. Erlenmeyer flask (*f*), which may be of glass, copper, or iron, is connected with the upright tube by a separate cork stopper.

Reagent. *Tetrachloroethane*, b.p. 144°.

Process. Place 60 ml. of the *tetrachloroethane* and 25 g. of the sample in the Erlenmeyer flask (*f*) and connect with the upright tube. Close stopcock *b* and introduce the condenser into *a*. Heat with a free flame which at first may be rather large. In about 30 seconds, when the tetrachloroethane begins to boil, regulate the flame so that the condenser delivers drops of moisture rapidly into the tube below. After about 20 minutes, when no more drops appear on the upright tube *d*, remove the flame. Allow to stand for a short time, then remove the condenser and rinse with *tetrachloroethane*. To remove drops of that liquid trapped in the water column, gently tap the tube with a glass rod.

Reading. Read the water column above the tetrachloroethane. If the column extends beyond the 15-ml. mark, incline the apparatus to the right and draw back into the flask through the stopcock a portion of the tetrachloroethane.

Calculation. To obtain the percentage of moisture, multiply the number of milliliters in the water column by 4. The proper limit for dried fruit is given as 30%.

Acids, Sucrose, Reducing Sugars, Fiber, Ash, Vitamins, Preservatives

See C3 above and D2 below.

2. CANDIED FRUIT; JUICES; SIRUPS; JELLIES; JAMS; MARMALADES; PRESERVES

There are two classes of sweetened fruit products: (1) those containing sufficient sugar to counteract acidity (candied fruits, fruit sirups, and canned fruits) and (2) those that are preserved by sugar or glucose (jellies, jams, and preserves). Those of the first class must be sterilized, those of the second class need not be sterilized, but should be protected from molds and other organized contaminants.[1]

The sweetening added to the product is more or less changed during processing by inversion or hydrolysis. The most important determinations are of solids, sugars, acids, vitamins, metals, artificial colors, and preservatives.

Solids

Since fruits and fruit products usually contain invert sugar in considerable, often large, amount, the directions for the following methods of the A.O.A.C. specify drying at 70° *in vacuo;* otherwise the levulose is decomposed.

Official A.O.A.C. Open Dish 70° Vacuum Method. In the absence of solid matter (fruit juices, sirups, jellies), employ the same method as for molasses and sugar sirup.

In the presence of solid matter (canned fruit, jams, marmalades, and preserves), employ an amount of the sample containing 3 to 4 g. of dry matter, add water if necessary to distribute the material, and dry *in vacuo* at 70° under a pressure not exceeding 100 mm. of mercury. Continue the drying until the loss in 2 hours does not exceed 3 mg.

Soluble Solids

Stevens and Baier Refractometric Method for Citrus Juices.[2] The method, devised at the California Fruit Growers Exchange, Ontario, Calif., involves no new chemical features, but supplies a table of corrections based on the percentages by weight of anhydrous citric acid, to be added to refractometric sucrose values derived from Schonrock's

or other table, to obtain the degrees Brix and the true percentage of soluble solids in citrus juices.

The intervals in the original table represent 0.2% by weight of anhydrous citric acid, but by interpolation the abridged table herewith may be used, since the true percentages of the anhydrous acid as shown by titration, when plotted as ordinates against corresponding refractometric sucrose values as abscissas, gives a practically straight line. The true percentages of the acid plotted against the degrees Brix give a line with a slight curve bending upward, but a 45° angle straight line drawn to represent the true percentage of soluble solids falls between the other two lines, although nearer the Brix line.

Corrections to Be Added to Refractometer Sucrose Values to Obtain Degrees Brix and Soluble Solids (Stevens and Baier)

Citric Acid %	Correction		Citric Acid %	Correction	
	Degrees Brix	Soluble solids		Degrees Brix	Soluble solids
1.0	0.20	0.11	16.0	3.00	1.97
2.0	0.39	0.23	17.0	3.17	2.10
3.0	0.58	0.35	18.0	3.35	2.23
4.0	0.78	0.47	19.0	3.53	2.35
5.0	0.97	0.59	20.0	3.70	2.47
6.0	1.15	0.71	21.0	3.88	2.59
7.0	1.34	0.84	22.0	4.05	2.73
8.0	1.54	0.96	23.0	4.24	2.84
9.0	1.72	1.08	24.0	4.41	2.97
10.0	1.91	1.21	25.0	4.58	3.10
11.0	2.10	1.33	26.0	4.76	3.23
12.0	2.27	1.46	27.0	4.94	3.35
13.0	2.46	1.59	28.0	5.10	3.48
14.0	2.64	1.72	29.0	5.28	3.60
15.0	2.81	1.84	30.0	5.46	3.73

The presence of an undue amount of water-insoluble solids in jams furnishes evidence of the removal of part of the juice for the manufacture of sirup or jelly, whereas a deficiency of insoluble solids indicates too small a proportion of fruit to sugar.

Kremla Direct Gravimetric Method.[3] This method, once official in the United States, directs to macerate 50 g. of the sample in a mortar with warm water, filter through muslin, wash with warm water with stirring up to 500 ml. or in extreme cases 1 liter. Transfer the residue to a dish, dry in a boiling water oven, and weigh.

German Indirect Gravimetric Method.[3] Weigh a suitable charge of the pulped sample into a graduated flask, shake well, make up to volume, and allow to settle. Decant the supernatant liquid, filtering if not clear. Evaporate an aliquot of the filtrate, dry at 70° in a boiling water oven, and weigh the soluble solids. Obtain the insoluble solids by difference.

Tentative A.O.A.C. Direct Gravimetric Method.[4] Weigh 25 g. of the pulped sample into a 400-ml. beaker and boil vigorously for 30 minutes with 200 ml. of water, replacing at intervals the water lost by evaporation. Filter on a weighed 5-inch (12.5-cm.) square of absorbent cotton, using a piece torn from the corner to plug the funnel. See that the pulp is loosened from the cotton with each addition. Use a total of 700 to 800 ml. of water. Fold the cotton over the insoluble matter, gently squeeze out the excess of water, and dry at 100° to constant weight.

Formic Acid

See Part I, C6*b*, and Vinegar below.

Tartaric Acid

Although not so widely distributed as some other organic acids, tartaric acid has long

been known to occur in large amount in the grape. It is not, however, the only acid. In the Concord grape, an American variety, Nelson, by the distillation method, found that about 60% of the acid was *l*-malic and only 40% *d*-tartaric acid and Hartmann and Hillig, by the pentabromacetone method, found small amounts of citric acid. The tartaric acid, whether optically active or racemic, exists partly free and partly combined as tartrates. See Part II, K2. The Hartmann and Hillig bitartrate method and the modified Kling racemate method which follow are tentative methods of the A.O.A.C. See also Hartmann Scheme below.

Hartmann and Hillig Bitartrate Volumetric Method.[5] Heczko[6] describes a modification of the Moszczenski method involving alkalimetric titration of the precipitated bitartrate and Kling and Florentin[7] devised a method involving titration with permanganate, both designed to supplant the then universal, but unsatisfactory, Goldenberg method. All these methods have given place to the Hartmann and Hillig method in the United States.

Reagent. *Lead Acetate Reagent.* Dissolve 75 g. of $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ in water acidulated with 1 ml. of acetic acid and dilute to 250 ml. with water.

Process. *Adjustment of Solution.* Determine the solids and acidity of the sample and weigh or measure a charge containing no more than 20 g. of solids and having an acidity no more than equivalent to 3 ml. of normal acid, designating as A the milliliters required. Adjust the volume of the charge to about 35 ml. by evaporation or dilution, add 3 ml. of *1.0 N sulfuric acid*, and heat at 50°. Transfer to a 250-ml. volumetric flask, rinsing with 10 ml. of hot water, followed by *ethanol*, cool, dilute to the mark with *ethanol*, and shake. Filter through a dry pleated paper and pipet 200 ml. of the filtrate into a centrifuge bottle.

If the sample contains ethanol, saponify, after adjusting to 35 ml., by adding $A + 3$ ml. of *1.0 N potassium hydroxide solution*, heating at 60°, and allowing to stand overnight. Add $A + 6$ ml. of *1.0 N sulfuric acid*, transfer to a 250 ml. volumetric flask, and proceed as above.

Lead Precipitation. To the 200 ml. of solution in the centrifuge bottle, add $A + 3$ ml., or if saponified $A + 6$, of *lead acetate reagent* and whirl at about 1000 r.p.m. for 15 minutes. Decant from the precipitate. If a precipitate forms on adding more of the reagent, shake and repeat the whirling. If the sediment lifts, repeat the whirling at a higher speed for a longer time. Allow to drain thoroughly and add to the precipitate in the centrifuge bottle 200 ml. of *80% ethanol*, shake vigorously, whirl, decant, and drain.

Removal of Lead. To the precipitate, add about 150 ml. of water, shake well, and saturate with *hydrogen sulfide gas*. Transfer to a 250-ml. volumetric flask, dilute to the mark with water, and filter through a dry pleated paper. Transfer 200 ml. of the clear filtrate to a 400-ml. beaker.

Ethanol Precipitation. Evaporate to 20 ml., neutralize to *phenolphthalein* with *1.0 N potassium hydroxide solution*, and add 5 drops in excess, then add 2 ml. of *glacial acetic acid* and 80 ml. of *ethanol* slowly with constant stirring. Chill in an ice bath for 2 minutes with vigorous stirring and store in the refrigerator overnight. Decant all the supernatant liquid but about 25 ml. onto a thin pad of asbestos in a Gooch crucible (best with a removable bottom). Add to the beaker about 0.3 g. of dry purified *asbestos*, mix well, and wash into the crucible with the cold filtrate. Finally rinse the beaker and contents of the crucible with three 15-ml. portions of ice cold *80% ethanol*, sucking dry after each.

Titration. Transfer the asbestos and precipitate to the original beaker with about 100 ml. of hot water, heat nearly to boiling, and titrate with *0.1 N alkali*, using *phenolphthalein indicator*.

Calculation. Use the equation: 1 ml. of 0.1 N alkali = 0.015 g. of tartaric acid.

Kling Racemate Volumetric Method.[8] Reagents. *Diammonium Citrate Solution.* Dissolve 29 g. of citric acid in about 200 ml. of water and carefully neutralize to methyl red with dilute NH_4OH, then add 14.5 g. of citric acid, dilute to 1 liter, and filter.

Ammonium Tartrate Solution. Dissolve in water 3.2 g. of the *l*-salt, free from the *d*-form, dilute to 200 ml., and filter. Add 1 ml. of formalin as a preservative.

Calcium Acetate Solution. Dissolve 16 g. of $CaCO_3$ in 120 ml. of glacial acetic acid diluted with water, dilute to 1 liter, and filter.

Calcium-Sodium Acetate Reagent. Dissolve 5 g. of $CaCO_3$ in 20 g. of acetic acid, add 100 g. of $NaC_2H_3O_2 \cdot 3H_2O$, dilute to 1 liter, and filter.

Standard Potassium Permanganate Solution. Dissolve 6.9745 g. of purest $KMnO_4$ in water and dilute to 1 liter. Titrate against a solution of pure tartaric acid as in the actual analysis; 1 ml. = nearly 0.005 g. of tartaric acid.

Standard Oxalic Acid Solution. Dissolve 13.8793 g. of purest oxalic acid in water and dilute to 1 liter. Titrate against the standard $KMnO_4$ solution.

Process. Proceed as in the Hartmann and Hillig bitartrate method up to the heading Ethanol Precipitation.

Calcium Racemate Precipitation. Evaporate the 200-ml. aliquot to about 100 ml. and add 50 ml. of water, 15 ml. of *diammonium citrate solution*, 25 ml. of *ammonium l-tartrate solution*, and 20 ml. of *calcium acetate solution*. Stir vigorously until the racemate begins to precipitate, then let stand overnight at room temperature. Decant through a thin, tightly tamped pad of asbestos in a Gooch crucible (best with removable bottom), and transfer the precipitate to the crucible with a portion of the filtrate. Wash the precipitate five times with water, filling the crucible half full and sucking dry each time.

Second Precipitation. Transfer the precipitate and mat from the crucible to a beaker, treat with 20 ml. of *1.5% hydrochloric acid*, and rinse the crucible with water. Add water to about 150 ml., then add 50 ml. of the *calcium-sodium acetate reagent*, and heat to about 80°. Cool, stir well, and let stand at least 4 hours with occasional stirring. Filter on a Gooch crucible, wash as before, and transfer pad and precipitate to a casserole, rinsing with 150 ml. of water. Add 50 ml. of *1 + 9 sulfuric acid* and heat to 80°.

Titration. Add immediately *standard potassium permanganate solution* in excess, again heat to 80°, add 5 ml. more of the *permanganate solution*, and allow to stand about 1 minute. Reheat to 80° and immediately add 10 ml. of *standard oxalic acid solution*, then titrate back with *permanganate solution*.

Calculation. Multiply the milliliters of permanganate solution required for the oxidation by 0.005 and divide the product by 2, thus obtaining the tartaric acid in the aliquot.

Citric Acid

See also Part II, G1.

Hartmann and Hillig Pentabromacetone Gravimetric Method.[9] Reagents. *Ferrous Sulfate Reagent.* Dissolve 40 g. of $Fe_2SO_4 \cdot 7H_2O$ in 100 ml. of water containing 1 ml. of H_2SO_4.

Lead Acetate Solution, 1.0 N. Dissolve 75 g. of $Pb(C_2H_3O_2) \cdot 3H_2O$ in water, add 1 ml. of glacial acetic acid, and dilute to 250 ml.

Potassium Bromide Solution. Dissolve 15 g. of KBr in 40 ml. of water.

Process. *Extraction.* Weigh out a quantity of the sample with titratable acidity not exceeding 3 ml. of normal acid and with solids content less than 20 g. Dilute or evaporate the portion so that it measures about 35 ml., add 3 ml. of *1.0 N sulfuric acid*, heat to 50°, transfer to a 250-ml. volumetric flask, rinsing

with 10 ml. of hot water, and then with *ethanol*. Cool, make up to the mark with *ethanol*, shake, filter through a dry paper, and pipet 200 ml. of the filtrate into a centrifuge bottle.

Saponification. If the sample contains ethanol, saponify with an amount of *1.0 N sodium hydroxide solution* equivalent to the acidity plus 3 ml. excess, heat at 60°, allow to stand overnight, and add an amount of *1.0 N sulfuric acid* equivalent to the original acidity plus 6 ml. excess.

Lead Precipitation. Add to the solution in the centrifuge bottle, *1.0 N lead acetate solution* sufficient to neutralize the original acidity plus 3 ml. of normal acid (or 6 ml. of the saponified solution), shake vigorously for 2 minutes, and whirl at 1000 r.p.m. for 15 minutes. Test the supernatant liquid with a few drops of the *lead acetate solution*, add more if necessary, shake, and whirl as before. Increase the speed and time of centrifuging if the precipitate is not obtained as a deposit that permits draining without loss.

Lead Removal. Drain well, add to the deposit in the bottle 200 ml. of *80% ethanol*, shake vigorously, whirl, decant, and drain. Add to the lead precipitate in the bottle 150 ml. of water, shake well, and saturate with *hydrogen sulfide gas*. Pour into a 250-ml. volumetric flask, rinse, fill to the mark with water, shake, and filter through dry paper.

Bromination. Pipet 200 ml. of the filtrate into a 500-ml. Erlenmeyer flask and gently boil down to about 75 ml. After cooling, add 10 ml. of *1 + 1 sulfuric acid* and 5 ml. of *potassium bromide solution*, heat to 48 to 50°, after 5 minutes add 50 ml. of *5% potassium permanganate solution*, and mix. Allow to stand 1 minute, stopper, shake for 1 minute, and allow to stand 3 additional minutes.

Add more *permanganate solution* if the test shows that it is not in excess. Begin the determination anew, using more *permanganate solution*, if the heavy precipitate of manganese dioxide disappears.

Pentabromacetone Precipitation. Add sufficient *ferrous sulfate reagent* (about 20 ml.) to dissolve the manganese dioxide, cool to 15°, stopper, shake vigorously, and keep in a refrigerator overnight. Filter as rapidly as possible on a Gooch crucible with thin, well-tamped asbestos mat, note the volume of the filtrate, then use it to transfer the precipitate to the crucible, wash without delay with 50 ml. of ice-cold water, dry by aspiration with thoroughly dried air introduced through a second Gooch crucible packed with cotton fitted to the top or by aeration as in the simplified method below.

Dissolve the pentabromacetone in three 20-ml. portions of *ethanol* and three 20-ml. portions of *ether*, dry, weigh the crucible, and deduct the weight from that of the crucible and pentabromacetone. The difference is the weight of the pentabromacetone.

Calculation. Calculate the milligrams of citric acid (C) in the aliquot by substituting in the following formula the weight of pentabromacetone in milligrams (P) and the volume of the filtrate in milliliters (V):

$$C = 1.05(0.424P + 0.017V) = 0.445P + 0.018V$$

The factor 1.05 corrects for the bulk of the lead precipitate, 0.017 is the weight of the pentabromacetone dissolved in each milliliter of filtrate, and 0.424 is obtained by dividing 192.11, the molecular weight of anhydrous citric acid, by 452.6, the molecular weight of pentabromacetone.

Hartmann Simplified Pentabromacetone Gravimetric Method.[10] Apparatus. *Knorr Filtering Tube*, 4.5 cm. long and 1.9 cm. in diameter with a stem 3.8 cm. long and two small "toes" to prevent rolling while weighing.

Aeration Train, for drying the pentabromacetone, consisting of 2 suction flasks containing sulfuric acid and a soda lime tower.

Reagent. *Iron Solution.* Dissolve 200 g.

of $FeSO_4 \cdot 7H_2O$ in water, dilute to 500 ml., add 5 ml. of H_2SO_4, and filter.

PROCESS. *Extraction. A. Jams and Preserves.* Mix the sample well and weigh 300 g. into a 2-liter volumetric flask. Dissolve in water, heating on the steam bath only to the extent necessary for solution. Cool, dilute to the mark, and filter. Pipet 200 ml. of the solution into a 600-ml. beaker and boil down to about 35 ml. Add, while still hot, *2 N sulfuric acid*, mix well, and let stand a few minutes. Pour into a 250-ml. volumetric flask and rinse the beaker with 15 ml. of hot water, followed by *ethanol*. Cool, dilute to the mark with *ethanol* and shake well. Adjust with *ethanol* to 20° and filter through absorbent cotton.

B. Jellies. Weigh 30 g. into a 250-ml. beaker, add 25 ml. of water, and boil down to 35 ml., then proceed as under *A*.

C. Fruit Juices. Transfer 20 g. with 30 ml. of water to a 250-ml. volumetric flask, add *2 N sulfuric acid,* and heat for 5 minutes on the steam bath. Cool and proceed as under *A*.

Isolation of Polybasic Acids. Determine the titer (t) of 10 ml. of the above filtrate in terms of milliliters of 0.1 N alkali. Pipet into a 250-ml. centrifuge bottle 200 ml. of the filtrate and add $0.03t \times (200/10)$ or, simplified, $0.6t$ g. of *powdered lead acetate* [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$], 0.03 g. being the weight that is about 50% more than equivalent to the combined acids. Stopper, shake vigorously for 5 minutes, and let stand 10 minutes with frequent shaking. Centrifuge at high speed, decant carefully, and reject the clear liquid. Add a little *saturated lead acetate solution*, if a test shows it to be necessary, and centrifuge again.

To the lead salts, add 50 ml. of *80% ethanol*, or 80 ml. of *95% ethanol*, and 20 ml. of water, and shake until the salts are completely dispersed. Fill the bottle with the *ethanol*, shake well, centrifuge, and reject the liquid, repeating once. Disperse the lead salts in 50 ml. of water, add 50 ml. more, and saturate with Transfer the solution to a 250-ml. flask, rinse with water, shake, fill to the mark, mix, and filter through a large pleated paper, pouring back until clear. Boil down exactly 200 ml. of the filtrate to about 50 ml.

Pentabromacetone Precipitation. Rinse the solution into a tared 300-ml. glass-stoppered Erlenmeyer flask and boil down to 40 g. Cool, add 2 g. of *potassium bromide* and 5 ml. of *sulfuric acid*, warm to 45 to 50°, and let stand a few minutes. Add immediately from a buret 25 ml. of *5% potassium permanganate solution* dropwise, swirl for 1 minute, and cool in the refrigerator for 30 minutes. Swirl again for 1 minute and add slowly with constant shaking the *iron reagent* until the solution starts to clear up. Shake vigorously and continue to add the *iron reagent* until the manganese dioxide is dissolved, then add a few milliliters in excess. Cool to 15°, add 20 g. of powdered *anhydrous sodium sulfate*, stopper, and shake for 3 minutes. Collect the pentabromacetone in a small Knorr filtering tube, washing the flask with a portion of the filtrate and the tube with about 50 ml. of water cooled to 15°.

Drying. Dry the precipitate over sulfuric acid in a vacuum desiccator overnight or by aeration as follows: wipe the inside of the tube with a small piece of filter paper and push it into the tube. Place the tube in the drying train, aerate 10 minutes, and weigh. Repeat the aeration for 5 minutes and reweigh, continuing the treatment until the loss does not exceed a few tenths of a milligram.

Dissolve the pentabromacetone in *ethanol* and *ether*, fill each tube three times with the solvent, reaerate, and weigh.

CALCULATION. Multiply the difference in the weights of the tube by 0.424 to obtain the grams of anhydrous citric acid in the aliquot.

NOTE. Rundell[11] adopted a procedure essentially the same as the Hartmann and Hillig method.

Kogan and Shtipel'man Acetone Iodometric Method.[12] The citric acid is oxidized with *potassium permanganate* in a phosphate buffered solution. The acetone thus produced is removed by distillation and titrated iodometrically. If acetaldehyde is formed during the process from lactic acid, tartaric acid, dextrose, or tannins, it may be destroyed in the distillate by oxidation with *potassium permanganate* in a sulfuric acid solution. After the excess of permanganate is reduced with *ferrous sulfate*, the acetone is distilled and titrated as above.

Pucher, Vickery, and Leavenworth Pentabromacetone Volumetric Method.[13] The procedure after the ether extraction combines certain features of the Kunz pentabromacetone method (Part II, F1), the Kretov, Panchenko, and Savich sodium sulfide dehalogenation method,[14] and the Volhardt silver nitrate-sulfocyanate halogen titration method.

APPARATUS. *Rubber Analysis Extraction Apparatus.* See Introduction, Apparatus.

REAGENTS. *Ferrous Sulfate Solution.* Dissolve 20 g. of $FeSO_4 \cdot 7H_2O$ in water, add 1 ml. of sulfuric acid, and dilute to 100 ml.

Hydrogen Peroxide, halogen-free. Dissolve 4 g. of Na_2O_2 in 50 ml. of water, cool carefully, and acidify faintly to Congo red with 1 + 1 H_2SO_4 (7 to 8 ml.). Prepare fresh each week.

Standard Silver Nitrate Solution, 0.05207 *N*. Dissolve 8.8462 g. of $AgNO_3$ and dilute to 1 liter; 1 ml. = 2 mg. of citric acid, or 4.714 mg. of pentabromacetone, or 4.162 mg. of bromine.

Ferric Alum Solution. Dissolve 100 g. of $FeNH_4(SO_4)_2 \cdot 12H_2O$ in water with warming and dilute to 100 ml. Warm slightly to dissolve the crystals before using.

Standard Ammonium Thiocyanate Solution, 0.05207 *N*. Dissolve 3.96 g. of NH_4SCN in water and dilute to 1 liter. Standardize against the silver nitrate solution.

PROCESS. *Ether Extraction.* Weigh out 2 g. of the sample and proceed as under the Pucher, Vickery, and Wakeman method for malic and citric acids below.

Citric Acid Oxidation. Pipet a suitable aliquot (usually 10 ml.), containing 1 to 20 mg. of citric acid, into a beaker, dilute to 25 ml., and add 3 ml. of *1 + 1 sulfuric acid.* Boil gently for a few minutes to expel traces of ether, cool, add 3 ml. of *bromine water*, and allow to stand 5 to 10 minutes. Filter through a Gooch crucible with gentle suction into a 150-ml. beaker bearing a mark at 40 ml. Wash the precipitate with small portions of water up to the 40-ml. mark, add 2 ml. of *1.0 M potassium bromide solution* and 10 ml. of *1.5 N potassium permanganate solution*, and allow the oxidation to proceed 10 minutes at room temperature. Cool to 10 to 15° in an ice bath, then add *ferrous sulfate reagent* (usually 20 to 30 ml.) until the manganese dioxide is dissolved.

Pentabromacetone Extraction. Transfer the oxidation mixture to a 125-ml. separatory funnel, previously cleaned with *chromic-sulfuric acid*, and rinse the beaker carefully with 25 ml. of *naphtha*, added in small portions. Shake vigorously and draw off the aqueous layer. Transfer the naphtha to a second separatory funnel. Shake the aqueous solution with 20 ml. of *naphtha* and combine the two naphtha extracts. Discard the aqueous extract. Wash the combined naphtha extracts four times with 3 to 4 ml. of water to remove inorganic halides.

Removal of Pentabromacetone and Dehalogenation. Add 5 ml. of freshly prepared *4% sodium sulfide solution* to the combined naphtha extract and shake thoroughly. The presence of pentabromacetone is indicated in the aqueous phase by a red color, closely proportional to the original citric acid content which is the basis for the Pucher, Sherman, and Vickery Method described below.

Draw off the aqueous layer into a 125-ml. Erlenmeyer flask and shake the naphtha layer with a second 5-ml. portion of *4%*

sodium sulfide solution, then three times with 3 to 4 ml. of water. To the combined aqueous extracts, add 20 ml. of water, 3 ml. of 2 *N sulfuric acid*, and a few angular *quartz pebbles*, then boil gently for 10 minutes to expel hydrogen sulfide. Cool, add *1.5 N potassium permanganate solution* (usually 1 to 3 ml.) until a red color persisting for 15 to 20 seconds appears. Decolorize with an excess of *halogen-free hydrogen peroxide*.

Silver Nitrate Precipitation. Add 5 ml. of *nitric acid* and 10 ml. of *standard 0.05207 silver nitrate solution*, then cool and stir, with the addition of a few drops of *ether*, to coagulate the silver bromide.

Thiocyanate Titration. Add 2 ml. of *ferric alum solution* and titrate with *standard 0.05207 N ammonium thiocyanate solution*, delivered from a micro pipet, to a faint salmon pink color.

CALCULATION. Multiply the number of milliliters of the standard silver nitrate solution required by 2×1.12 to obtain the weight of citric acid in the aliquot of the organic acid solution taken.

Pucher, Sherman, and Vickery Penta-Bromacetone Colorimetric Method.[15] This method, employing certain features of the Pucher, Vickery, and Leavenworth Volumetric Method (above), was designed for the determination of small amounts of citric acid (0.1 to 1 mg.) in animal and vegetable products.

APPARATUS. *Pulfrich Photometer*, with cell and light filter S 43.

REAGENTS. *Citric Acid*. Stock solution: establish the purity of the acid by titration and prepare in 1.0 N H_2SO_4 a solution of which 1 ml. is equivalent to 10 mg. of anhydrous citric acid.

Ferrous Sulfate Solution, 20% in 1% H_2SO_4.

Pyridine. Redistil Eastman's Practical Grade, b.p. 112 to 117°.

PROCESS. *Preliminary Treatment and Oxidation*. Transfer an aliquot, containing not over 1 mg. of citric acid, to a beaker, add water to about 75 ml., then 3 ml. of *50% sulfuric acid* and a few angular *quartz pebbles*. After boiling about 10 minutes, to decompose any unknown substances which later would have combined with the bromine, the solution should measure about 40 ml. Cool to room temperature in a refrigerator, add an excess of *saturated bromine water* (usually 3 ml.) and allow to stand 10 minutes. Should a precipitate form, allow to stand 20 minutes longer, adding more *bromine water* from time to time to insure an excess. Transfer to a 50-ml. centrifuge tube, centrifuge, and pour the supernatant liquid into a 125-ml. pear-shaped separatory funnel, rinsing being unnecessary. Add 2 ml. of *1 M potassium bromide solution* and 10 ml. of *1.5 N potassium permanganate solution*, allow to stand 10 minutes, and decolorize with the requisite amount of *ferrous sulfate reagent*.

Pentabromacetone Extraction. Shake the mixture with 25 ml. of *naphtha*, draw off the aqueous layer, and wash the naphtha once with 5 to 10 ml. of water, adding the wash fluid to the aqueous solution. Transfer the naphtha solution to a second separatory funnel, return the aqueous solution to the first funnel, and reextract, adding the second naphtha extract to the first. Wash the combined extracts four tines with 5-ml. portions of water.

Color Formation. Shake the washed naphtha successively with 3-, 2-, and 1-ml. portions of filtered *4% sodium sulfide solution*, drawing off into 10-ml. volumetric flasks containing 3.5 ml. of *pyridine*, and make up to volume with *50% pyridine solution*.

Color Reading. Determine the extinction coefficient within 30 minutes in the Pulfrich photometer, using a cell of appropriate length and light filter S 43. Place water in the control cell.

CALCULATION. Derive the milligrams of citric acid from a calibration curve prepared

from the results obtained on a series of solutions, containing from 0.1 to 1 mg. of *citric acid*, omitting the preliminary boiling and treatment with bromine.

NOTE. Josephson and Forssberg [16] found that pure *glycerol* is a better color stabilizer than pyridine. The extinction coefficient is directly proportional to the concentration of citric acid when using light filter S 47 instead of S 43.

MALIC ACID

Muttelet Barium Bromide Gravimetric Method.[17] Muttelet, as a means of detecting apple, cherry, and other large-fruit pulp or juice in small-fruit jams and jellies, determines malic acid, which is present only in small amount, if at all, in the juice of currants, gooseberries, raspberries, and strawberries. In addition to the apple as well as other pomes and the cherry, the plum contains malic acid in predominating amount and this fruit, rather than the cherry, is likely to be substituted in jams for the small fruits. There is great variation in the actual and relative amounts of malic acid due to variety, stage of ripening, and other causes and there is some conflicting evidence. The Muttelet method, although defective, has considerable practical and scientific value.

PROCESS. Make all dilutions and precipitations at 15°.

Clarification with 1 + 1 Ethanol. Macerate well 100 g. of the sample with 50 ml. of water in a porcelain dish, heat on a water bath, and transfer to a 300-ml. volumetric flask. Continue the heating with shaking until all the soluble matter has dissolved, then cool and add slowly with shaking 150 ml. of *ethanol*, make up to the mark with water, mix, let stand a few hours, and filter through a dry paper. If the filtrate is highly colored, shake with 3 to 4 g. of *alkali-free charcoal* and, after 15 to 20 minutes, again filter.

Acidity Determination. Titrate a 20-ml. aliquot of the solution, diluted to 100 ml., with *0.1 N potassium hydroxide solution*, using *phenolphthalein indicator*.

Barium Bromide Precipitation in 2 + 1 Ethanolic Solution. To a 100-ml. aliquot of the filtrate add 200 ml. of *ethanol*, neutralize with *1.0 N potassium hydroxide solution*, then add 25 to 30 ml. of *5% barium bromide solution in 85% ethanol* and allow to stand for a few hours. Filter and wash the precipitate with *80% ethanol* until the excess of barium has been removed.

Barium Citrate Precipitation in 1 + 2 Ethanolic Solution. Transfer the precipitate to an Erlenmeyer flask, dilute to 100 ml., digest on the water bath with water for 1 hour, cool, filter, and add 50 ml. of *ethanol* slowly with stirring. Collect the barium citrate on a filter and wash with *1 + 2 ethanol.* Evaporate the filtrate to 50 ml., cool, and add 25 ml. of *ethanol.* Filter to remove the last traces of barium citrate. Concentrate the filtrate on a water bath to 25 ml. and filter if a precipitate forms.

Barium Malate Precipitation in 2 + 1 Ethanolic Solution. Add 2 volumes of *ethanol* to the combined 1 + 2 ethanolic filtrates. Filter and wash the precipitate with 66% ethanol.

Barium Sulfate Precipitation. Dissolve the barium malate in water, precipitate with *sulfuric acid*, filter, wash, ignite, and weigh the barium sulfate in the usual manner.

CALCULATION. To obtain the weight of malic acid, multiply the weight of barium sulfate by 0.574.

Espeso Modification.[18] Miss Espeso makes no material changes in the main process, but emphasizes the importance of the complete removal of occluded malic acid from the barium citrate precipitated in the 1 + 2 ethanolic solution and of the verification that the precipitate formed in the 2 + 1 ethanolic solution is indeed pure barium malate. Malic acid is detected by the Pinerna test, using the reagent prepared by dissolving 0.2

g. of *β-naphthol* in 1 ml. of *sulfuric acid.*[19] In the presence of barium malate, a green fluorescence is obtained on heating a small fraction of the precipitate with 1 ml. of the *β-naphthol reagent.*

She appears also to have determined the citric acid in the barium citrate precipitate by precipitation of barium sulfate in a manner analogous to that given by Muttelet for the determination of malic acid in the barium malate precipitate, thus extending the application of the method.

Levo Malic Acid

Hartmann and Hillig Uranium Acetate Polariscopic Method. See Hartmann Scheme below.

Inactive (Racemic) Malic Acid

Hartmann and Hillig Permanganate Oxidation Polariscopic Method. See Hartmann Scheme below.

Malic and Citric Acids

Pucher, Vickery, and Wakeman Dinitrophenylhydrazine Colorimetric-Volumetric Method. See Hartmann Scheme below.

Hartmann Scheme for Polybasic Organic Acids[20]

The scheme is a notable contribution to fruit chemistry. All steps were checked on known mixtures.

Occurrence. The organic polybasic acids occurring naturally in the common fruits are: (1) *levo malic* and *normal citric* (almost universal), (2) *isocitric* (in blackberry and its hybrids), (3) *tartaric* (with certainty only in the grape and the tamarind), (4) *tannic* (in all fruits), and (5) *oxalic* and *succinic* (sparingly in the fresh common fruits; *succinic* in considerable amount in some fermented beverages). Practically all the acid of apples, quinces, plums, prunes, and ries is malic and that of citrus fruits is citric. The grape contains malic, as well as tartaric. Excepting the grape and the blackberry, natural fruits contain neither tartaric nor isocitric acid and malic acid may be determined directly in the isolated acid solution. *Inactive (racemic) malic acid* occurs solely as an added ingredient.

All the acids named are readily soluble in ethanol and precipitated as the lead salts from strong ethanol. *Pectin* (pectic acid) occurs in all fruits; it is characterized by precipitation by strong ethanol from weak acid solution.

Characteristic Reactions. *Pectic, tartaric, levo malic, tannic,* and *isocitric acids* are optically active; *inactive malic, normal citric, succinic,* and *oxalic acids* are inactive. Only *tartaric acid* forms an ethanol-insoluble potassium hydrogen salt. Only *normal citric acid* yields pentabromacetone. Only *succinic acid* resists oxidation with potassium permanganate. In neutral solution, only *tannic acid* is quantitatively separated by adsorption on carbon. *Levo* and *inactive malic acids,* also *normal citric, isocitric,* and *tannic acids* are oxidized in the presence of potassium bromide, forming volatile bromine compounds yielding insoluble condensation products with dinitrophenylhydrazine. (See Pucher, Vickery, and Wakeman below.) *Lead* and *calcium oxalates* are practically insoluble in weak acetic acid solution. *Isocitric acid* yields with uranium acetate a dextrorotatory complex which is removed by lead acetate in 2.5% acetic acid solution, permitting determination of *levo malic acid.*

Process. *Extraction.* Boil gently for 1 hour 50 g. of fresh fruit, or 10 g. of dried fruit, with 150 ml. of water, replacing the loss through evaporation. Cool, dilute to 250 ml. in a volumetric flask, and filter through absorbent cotton.

Removal of Pectin. Boil down a 200-ml. aliquot to about 60 ml., add while hot 2 ml.

of *1.0 N nitric acid* to liberate the acids, mix, transfer to a 250-ml. volumetric flask, rinse with 10 ml. of hot water, followed by *ethanol.* Cool, dilute to the mark with *ethanol* at 20°, and filter through cotton forming a lining on the funnel. To increase the speed of filtration, bring the ends of the cotton together and squeeze.

Isolation of Polybasic Acids. Using *phenolphthalein indicator*, titrate 10 ml. of the filtrate with *0.1 N sodium hydroxide solution* (titer = t), then to 200 ml. of the filtrate add $2t + 2$ ml. of *1.0 N sodium hydroxide solution* and heat on the steam bath for 30 minutes to saponify the esters. Cool under the tap, add 5 ml. of *1.0 N acetic acid* to hold lead salts of monobasic acids in solution, and rinse with *ethanol* into a 250-ml. centrifuge bottle. Add $0.6t$ g. of *finely powdered lead acetate,* shake vigorously for 5 minutes, then add 0.2 g. of *Filter-Cel,* fill with *ethanol,* and mix well. Centrifuge 5 minutes at about 1000 r.p.m., decant the supernatant liquid, and discard. Add to the residue 50 ml. of *80% ethanol* and shake until the lead salts are completely dispersed. Fill the bottle with *80% ethanol,* mix well, and centrifuge. Discard the supernatant liquid and again add *ethanol* and centrifuge, then disperse the lead salts in 50 ml. of water, dilute to 150 ml., and saturate with *hydrogen sulfide gas.* Shake about 1 minute, rinse into a 250-ml. volumetric flask, fill to mark, and filter through a large pleated paper, pouring back until clear.

In aliquots of the filtrate (F) determine tartaric, normal citric, levo malic, inactive malic, and isocitric acids, and in a fresh portion of the sample determine tannin and colors as follows.

I. Tartaric Acid

Modified Hartmann and Hillig Bitartrate Volumetric Method. The original method [21] has been modified by Hartmann [22] as follows.

Process. *Titration.* Dilute 20 ml. of filtrate F (above) with 50 ml. of water and expel the hydrogen sulfide by boiling for a few minutes. Cool, titrate with *0.1 N sodium hydroxide solution* (titer = t). Boil down 200 ml. of filtrate F to about 20 ml., cool, rinse into a tared 300-ml. glass-stoppered Erlenmeyer flask, and adjust with water to about 40 g.

Precipitation of Potassium Bitartrate. Add 0.0940 g. of purest *Rochelle salt* (= 50 mg. of tartaric acid), $0.2t$ g. of *potassium acetate,* 1 ml. of *glacial acetic acid,* and 160 ml. of *ethanol.* Cool under the tap, add a dozen glass beads, and shake vigorously for 5 minutes. Cool in the refrigerator for 15 minutes and shake for 1 minute, then again cool for 15 minutes, shake for 1 minute, and immediately filter through asbestos in a Gooch crucible. Rinse the flask with about 100 ml. of cold *ethanol,* pour the rinsings through the crucible, and continue the suction for 5 minutes after the liquid has run through. Set aside the filtrate (F').

Titration. Rinse the crucible and flask with hot water into a beaker and titrate with *0.1 N sodium hydroxide solution* (titer = T).

Calculation. Obtain by the following formula the grams of tartaric acid (G) in the portion taken for analysis:

$$G = \frac{0.015T - 0.050}{0.512}$$

in which $0.015T$ and 0.050 are grams of tartaric acid present and added respectively and 0.512 is the dilution of the final aliquot.

II. Normal Citric Acid

Modified Hartmann and Hillig Pentabromacetone Gravimetric Method. The original method [23] has been modified by Hartmann [24] as follows.

Process. Boil down 200 ml. of filtrate F (above) to about 20 ml., rinse into a 300-ml. glass-stoppered Erlenmeyer flask, and adjust with water to about 40 g.

Bromination. Add 2 g. of *potassium bromide* and 5 ml. of *sulfuric acid*, heat to about 50°, and allow to stand 5 minutes.

Formation of Pentabromacetone. Add 20 ml. of *5% potassium permanganate solution* from a pipet slowly in 1- or 2-ml. portions, swirling the flask a few seconds after each. Let stand undisturbed 5 minutes and cool to 15°. Add slowly with constant agitation *40% ferrous sulfate solution in 1% sulfuric acid* until the mixture starts to clear up. Shake 1 minute, then continue the addition of the *iron solution* until the manganese dioxide is dissolved and add a few milliliters in excess. Add 20 g. of *anhydrous sodium sulfate*, cool to 15°, and shake vigorously 5 minutes. Immediately collect the pentabromacetone on asbestos in a Gooch crucible and rinse the precipitate from the flask with a portion of the filtrate. Wash the precipitate with 50 ml. of cold water and continue the suction for a few minutes after the liquid has run through. Dry overnight in a sulfuric acid desiccator and weigh (a). Remove the pentabromacetone from the crucible with *ethanol* followed by *ether*, filling the crucible three times with each solvent. Dry 10 minutes in the water oven, place in the desiccator 5 minutes, and weigh (b).

CALCULATION. Obtain the grams of anhydrous citric acid (G) in the portion taken for analysis by the following formula:

$$G = \frac{0.424(a-b)}{0.512}$$

in which 0.424 is the factor for converting pentabromacetone into anhydrous citric acid, $a - b$ is the weight of pentabromacetone in the aliquot, and 0.512 is the dilution of the final aliquot.

III. LEVO MALIC ACID

Modified Hartmann and Hillig Uranium Acetate Polariscopic Method. The original method [25] has been modified by Hartmann [26] as follows.

PROCESS. *Removal of Isocitric Acid by Lead Treatment.* Rinse filtrate F' (above) with a small quantity of *ethanol* into a 500-ml. Erlenmeyer flask and add $0.03\ (10t - 2T + 6.6)$ g. of *lead acetate* dissolved in a few milliliters of water. (See Tartaric Acid above.) Shake vigorously 5 minutes, add 0.3 g. of *Filter-Cel*, and mix well. Fill a centrifuge bottle, mix, centrifuge, discard the supernatant liquid, add the remainder of the mixture, rinse the flask with about 40 ml. of *ethanol*, and fill the centrifuge bottle with *80% ethanol*. Mix well, again centrifuge, and discard the liquid. Disperse the salts in 50 ml. of *80% ethanol* and fill the bottle with *80% ethanol*. Mix well, centrifuge, and discard the liquid, then again repeat the addition of *ethanol* and *centrifuge*. Completely disperse the lead salts in water and dilute to about 150 ml. Saturate with *hydrogen sulfide gas*, shake 1 minute, and transfer to a 250-ml. volumetric flask. Fill to the mark and filter through a large pleated paper, pouring back until clear.

Boil down 200 ml. of the filtrate to about 10 ml. and with 20 ml. of water rinse into an accurately tared 50-ml. Erlenmeyer flask. Cool under the tap, add about 0.05 g. of *citric acid*, and neutralize to *phenolphthalein* with *1.0 N sodium hydroxide solution* (t). To the neutralized solution, add 1 ml. of *glacial acetic acid* and 1 g. of *sodium acetate*. Add $0.25t$ g. of *lead acetate* and adjust with water to 40 g. (±0.2 *g.*). Shake the mixture 5 minutes and let stand 10 minutes.

Uranium Acetate Treatment. Filter through a small pleated paper into a small Erlenmeyer flask and add 2 g. of *uranium acetate*. Shake the mixture vigorously 5 minutes, add 0.5 g. of *carbon*, again shake 3 minutes, and let stand 10 minutes, shaking occasionally. Filter through a small pleated paper into a 200-mm. polariscope tube, discarding the first few milliliters of filtrate and pouring back until clear.

Polarization. Make at least five readings

at 20°, using white light, and take the average. If control for adjusting to standard temperature is lacking, determine the temperature of the polariscope and prepare the solution of the uranium complex at this temperature. Make readings after allowing the tube to remain in the instrument trough for 30 minutes.

Calculation. Obtain the grams of levo malic acid (G) in the portion taken for analysis by the following formula:

$$G = \frac{0.0185V}{0.41}$$

IV. Inactive (Racemic) Malic Acid

Modified Pucher, Vickery, and Wakeman Dinitrophenylhydrazine Method. The original photometric method [27] was designed for tobacco. The following gravimetric procedure is Hartmann's adaptation [28] combined with certain features of the Hartmann and Hillig Permanganate Oxidation Polariscopic Method (above).

Process. (a) *Total Hydrazine Precipitate.* Treat the filtrate F' under Tartaric Acid (above) as directed under Levo Malic Acid (above) up to and including "Add 0.25t g. of lead acetate" in the third paragraph. Shake the mixture 5 minutes, allow to stand 10 minutes, add 0.5 g. of *carbon,* and shake 3 minutes. Filter into a 50-ml. flask, pouring back until clear. Drain thoroughly. Pipet 10 ml. of the filtrate into a 300-ml. glass-stoppered Erlenmeyer flask and set the remaining filtrate (F'') aside for the Malic Acid Polarization (below).

Bromination. To the 10-ml. filtrate in the Erlenmeyer flask, add about 30 ml. of water and 0.05 g. of *citric acid,* then 0.5 g. of *potassium bromide* and 3 ml. of *sulfuric acid,* heat to about 50°, and let stand 5 minutes.

Oxidation. Add 10-ml. of *5% potassium permanganate solution* from a pipet 1 to 2 ml. at a time, swirling after each addition. Let stand 5 minutes, cool to 15°, then add *3% sodium sulfite solution* (dropwise toward the end) until the manganese dioxide is just dissolved, avoiding a large excess. Again add the *potassium permanganate solution* until a slight but distinct brownish precipitate of manganese dioxide is produced. Cool to 15°, add 20 g. of *anhydrous sodium sulfate,* and shake vigorously 5 minutes. Filter on asbestos in a Gooch crucible and wash with about 70 ml. of cold water. Rinse the filtrate into a 400-ml. beaker and add the *sodium sulfite solution* dropwise until the solution is decolorized. Add 15 ml. of *hydrochloric acid,* mix well, then add immediately a *solution of 0.5 g. of 2,4-dinitrophenylhydrazine in 20 ml. of water and 10 ml. of hydrochloric acid,* previously heated to boiling and filtered. Bring to a boil and let stand on the steam bath for 30 minutes with stirring. Immediately filter on asbestos in a weighed Gooch crucible and wash with 100 ml. of hot *dilute hydrochloric acid* (5 + 100). Dry the crucible 30 minutes in the water oven and weigh.

(b) *Malic Acid Polarization.* To the remainder of filtrate F'' in the 50-ml. flask, add 2 g. of *uranium acetate* and shake vigorously 5 minutes. Add 0.1 g. of *Filter-Cel,* mix well, and filter into a dry 200-mm. polariscope tube, pouring back until clear. Polarize at 20°, using white light.

Calculation. Obtain the grams of inactive malic acid (G) in the portion taken for analysis by the following formula:

$$\frac{3.28P - 0.0185V°}{0.41}$$

derived from

$$\frac{0.0185}{0.0156}$$

in which 0.0185 is the polarization value under the conditions of analysis and 0.0156 is the polarization value of malic acid in pure solution, 0.712 is the hydrazine value of malic acid, P is the grams of hydrazine pre-

cipitate, $V°$ is the polariscopic reading, and 10.3 is the average weight of the 10-ml. solution taken for the hydrazine precipitation.

V. Isocitric Acid

Hartmann Polariscopic Method.[29] Process. The acid is obtained by the difference of polarization readings.

(*a*) *Rotation of Isocitric Acid Plus Levo Malic Acid.* Treat the filtrate *F′* obtained in the determination of tartaric acid as directed for the determination of Levo Malic Acid (above) in the entire paragraph beginning "Removal of Isocitric Acid by Lead Treatment."

Uranium Acetate Treatment. Pipet 200 ml. of the filtrate into a 400-ml. beaker and boil 5 minutes to expel hydrogen sulfide. Neutralize to *phenolphthalein* with *1.0 N sodium hydroxide solution* and add 0.5 ml. in excess. Boil down to about 10 ml. and rinse with 20 ml. of water into a tared 50-ml. Erlenmeyer flask. Add 5 drops of *glacial acetic acid* and adjust with water to 40 g. (±0.2 g.). Add 2 g. of *uranium acetate* and shake vigorously 5 minutes. Add 0.5 g. of *carbon*, shake 3 minutes, and let stand 10 minutes, shaking occasionally. Filter through a small pleated paper into a 200-mm. polariscope tube, pouring back until clear, and read at 20° with white light (*a*).

(*b*) *Rotation of Levo Malic Acid.* Determine the rotation (*b*) of levo malic acid as directed under that head above.

Calculation. Obtain by the following formula the grams of isocitric acid (G) in the portion taken for analysis:

$$G = 0.03(a - 1.19b)$$

in which 0.03 is rotation value of isocitric acid, a is optical rotation of isocitric + malic acids under conditions of analysis, and $1.19b$ is correction for rotation of malic acid.

VI. Tannin and Colori

Direct treatment with carbon as directed for wines (Part II, F1) gives high results owing to the adsorption of free acids. By neutralizing with calcium carbonate, the tannin may be quantitatively separated from the other acids. The oxidizable matter is determined in the neutralized solution before and after treatment with carbon and the tannin plus coloring matter is calculated by difference in terms of tannic acid ($C_{14}H_{10}O_9$).

Reagents. *Standard Potassium Permanganate Solution.* Dilute 576 ml. of 0.1 *N* $KMnO_4$ solution to 1 liter; 1 ml. = 0.002 g. of $C_{14}H_{10}O_9$.

Solution. Dissolve 6 g. of sodium disulfonate in 500 ml. of water by heating, cool, add 50 g. of H_2SO_4, dilute to 1 liter, and filter.

Process. *Extraction.* Weigh 50 g. of a light-colored fruit (apple, peach, pear, etc.) or 20 g. of a deeper-colored fruit (blackberry, raspberry, strawberry, etc.) into a 500-ml. beaker, add 300 ml. of water, and boil gently 1 hour, replacing at intervals the water evaporated. Cool, transfer to a 500-ml. volumetric flask, and dilute to the mark. Mix well and filter through cotton. Dealcoholize fermented beverages.

Neutralization. Pipet 400 ml. of the filtrate into a 600-ml. beaker, add 0.3 g. of *powdered calcium carbonate*, and heat to boiling. Cool, dilute to the mark in a 500-ml. volumetric flask, add 0.5 g. of *Filter-Cel*, mix well, and filter through a large pleated paper, pouring back until clear.

(*a*) *Direct Permanganate Titration.* Pipet 200 ml. of the filtrate into a 2-liter porcelain dish, add about 800 ml. of tap water and exactly 20 ml. of the *indigo solution.* Add *standard potassium permanganate solution*, 1 ml. at a time, stirring vigorously until the blue color changes to green, then add dropwise until the color becomes golden yellow.

(*b*) *Permanganate Titration after Clarifi

tion. To the remaining filtrate, add 1 g. of *sensitized carbon*, shake a few minutes, and let stand 30 minutes, shaking frequently. Filter through a large fast-flowing pleated paper, pouring back until clear. Pipet 200 ml. into the porcelain dish and add 800 ml. of tap water and 20 ml. of the *indigo solution*. Titrate with *standard potassium permanganate solution* as above.

CALCULATION. Obtain by the following formula the grams of tannic acid (G) in the portion taken for analysis:

$$G = \frac{0.002(b - c)}{0.32}$$

in which 0.002 is gram of $C_{14}H_{10}O_9$ per milliliter of 0.1 N $KMnO_4$, b is the milliliters of $KMnO_4$ required in the direct titration, and c is the milliliters of $KMnO_4$ used in the titration of the clarified solution.

SUCROSE; REDUCING SUGARS; COMMERCIAL GLUCOSE

See Part I, C6*a* and Part II, E2 and E3.

PECTIN

(Alcohol Precipitate)

The term pectin (pectic acid of other authors) is applied by Norris and Schryver [30] to a substance formed by the union of 1 galactose, 1 arabinose, and 4 galacturonic acid groups and pectinogen (pectin of some other authors) to the propectin which has in addition a methyl group together with calcium and other bases. Other authors believe the acetyl group is present in pectinogen. Pectin by reason of its jellying property is of great nutritional significance, since it permits the storing of the desirable qualities of fruit in the form of jams and jellies.

The methods for the determination of alcohol precipitate and pectic acid (pectin) tentatively adopted by the A.O.A.C. are of economic importance in the fruit products industry and in the inspection of the commercial products.

Ethanol Precipitation Gravimetric Method.[31] PROCESS. *Solution.* Boil for 1 hour 300 g. of the pulped fruit, jelly, or fruit sirup with 800 ml. of water, cool, dilute to 2 liters, and filter.

Ethanol Precipitation. To an aliquot of 200 ml. in a beaker, add 8 to 12 g. of *sugar*, if not already sweetened, and evaporate to 25 ml. If organic acids are to be determined in the filtrate from the pectin, cool, add 3 ml. of *1.0 N sulfuric acid*, followed immediately by 200 ml. of *ethanol* with constant stirring. If organic acids are not to be determined, omit the addition of sulfuric acid. In either case, allow the precipitate to settle, filter on a 15-ml. quantitative paper, and wash with *ethanol*.

First Reprecipitation. Transfer the precipitate to the original beaker with hot water, evaporate to about 40 ml., stirring vigorously during the evaporation with the addition of a few drops of *1 + 2.5 hydrochloric acid* if a precipitate forms, and cool to 25° or below. Add 50 ml. of *sodium hydroxide solution*, containing 2 to 5 ml. of *10% sodium hydroxide solution*, depending on the volume of the precipitate, and allow to stand 15 minutes. Add 40 ml. of water and 10 ml. of *1 + 2.5 hydrochloric acid*, and boil 15 minutes. Filter rapidly and wash the precipitate of pectic acid with hot water. If the filtrate is not clear, start afresh, using more *alkali* and saponifying at a lower temperature.

Second Reprecipitation. Wash the precipitate back into the beaker, adjust to 40 ml., cool below 25°, repeat the saponification with *alkali*, the precipitation with *acid*, and the boiling and washing as before, but wash only until the filtrate does not exceed 500 ml. and is only slightly acid. Wash the precipitate into a tared platinum dish, dry on the steam bath, then in a boiling water oven to constant weight. Ignite and weigh again.

CALCULATION. The loss in weight is pectic acid.

NOTE. The tentative method for the determination of alcohol precipitate is essentially the same as the above except that only one reprecipitation is made.

SORBITOL

See Part II, F1.

NARINGIN

Harvey Ferric Chloride Colorimetric Method.[32] APPARATUS. *Colorimeter.*

PROCESS. Prepare a hot water extract of the grapefruit rind, add *ferric chloride solution*, and compare in a colorimeter with a *standard naringin solution* treated in like manner.

RIBOFLAVIN

See Part II, C1, Mackinney and Sugihara Modification of the Conner and Straub Decalso-Supersorb Fluorometric Method.

EXAMPLES (Miss M. B. Smith). Apricots, dried, sulfured 0.21; prunes, dried 1.24; and dates, Deglet Noors 0.52 to 0.53 γ/g.

ASCORBIC ACID

Indophenol Method. See Part I, C10.

Bennett[33] notes that the interference of sulfurous acid, added to fruit juices as a preservative, can be avoided by boiling and rapidly cooling prior to titration.

Dilute 2 ml. of the juice with water in a small flask, place a funnel in the neck, and boil for 2 minutes. Cool rapidly under the tap and titrate immediately. The loss of the vitamin sustained when pure juice is boiled, due to dissolved oxygen, does not take place in sulfured juice.

Downer[34] removes sulfurous acid by distillation of the deaerated juice with *5% phosphoric acid* in a current of *carbon dioxide.*

Kirkpatrick[35] distils with steam into *hydrogen peroxide*, using the residue for ascorbic acid titration and the distillate for sulfurous acid determination by titration with *sodium hydroxide solution.*

Lorenz-Stevens Iodometric Method. Since iodine titration was first proposed by Lorenz, Reynolds, and Stevens[36] the method has been extensively used by the California Fruit Growers Exchange and others because of its rapidity, stability of the reagents, sharpness of the end-point, agreement of duplicates, and low cost of reagents. It has been shown, however, by Stevens[37] that in its original form it is less specific than the 2,6-dichlorophenol-indophenol method and by Di Gléria[38] that the results are often 20% higher. Among others who have used the method are Mack, Fellers, Maclinn, and Bean,[39] Roberts,[40] and Buogo.[41]

The directions which follow are those given by Stevens[37] who by special manipulation has eliminated the most serious errors of the process.

REAGENTS. *Standard Iodine Solution*, 0.01 *N*. Prepare a stock solution of approximately 0.1 *N* by dissolving 25 g. of KI in the smallest possible amount of water, adding 12.7 g. of resublimed iodine, and diluting to 1 liter. Keep in a dark bottle. As needed, dilute about 100 ml. of the stock solution together with 22.5 g. of KI to 1 liter. Standardize by titrating 20- to 25-ml. portions against standard 0.01 *N* $Na_2S_2O_3 \cdot 5H_2O$ solution, adding 3 ml. of starch solution as indicator toward the end of the operation.

Starch Solution, 0.5%. Prepare as directed by Treadwell and Hall[42] as follows. Make a paste by rubbing up 5 g. of soluble starch with a little cold water and pour into 25 ml. of boiling water. Boil for about 2 minutes, cool, and add 1 g. of KI. Use 0.5 ml. for each 100 ml. of solution.

Sodium Thiosulfate Solution, 0.01 *N*. Stevens prefers the Bray and Miller[43] technique of titration against $K_2Cr_2O_7$.

PROCESS (for Citrus Juices). *Titration.* Mix 20 ml. of the natural strength juice in a 250-ml. Erlenmeyer flask with 4 ml. of *12 N sulfuric acid*, thus lowering the *p*H, as determined by the hydrogen electrode, to between 0.02 and 0.08. Add freshly standardized *0.01 N iodine solution* until the excess is 1 to 2 ml., as determined by the color change of the sample alone or with an added drop of *starch solution.* After 30 seconds, add 3 ml. of the *starch solution* and *standard 0.01 N thiosulfate* to an excess of about 1 ml. Finally titrate back slowly with standard iodine solution.

CALCULATION. Subtract from the total volume of iodine solution added the volume of the thiosulfate (in terms of the iodine solution if not strictly 0.01 *N*), thus obtaining the volume of iodine solution consumed; 1 ml. of 0.01 *N* iodine solution ≐ 0.88 mg. of ascorbic acid.

Scudi and Ratish Diazotized Sulfanilamide Colorimetric Method.[44] The authors (Harlem Hospital, New York City) obtained by this method results on citrus juices showing reasonable agreement with those by the dichlorophenol-indophenol method. Cysteine, tyrosine, histidine, creatinine, ammonia, phenol, and uric acid do not influence the results, but hydroquinone and gluco-reductones interfere, although by coupling in acid media the interference is reduced.

REAGENT. *Dimethylnaphthylamine Solution.* Dilute 1 ml. of 1-dimethylnaphthylamine (Eastman No. 1063) to 500 ml. with ethanol.

PROCESS. *Color Formation.* Mix 5 ml. of *0.005% sulfanilamide solution*, 1 ml. of *0.05% sodium nitrite solution* (prepared daily), and 1 ml. of *20% sulfosalicylic acid solution*, allow to stand 1 to 3 minutes, and add 1 ml. of *1% urea solution.* After 5 minutes, add 10 ml. of a freshly prepared extract of the sample containing 0.1 to 0.4 mg. of the vitamin in *10% acetic acid*, then after another interval of 5 minutes add 7.4 ml. of *0.2% 1-dimethylnaphthylamine solution in ethanol*, and mix.

Color Comparison. Compare the color developed between 10 and 50 minutes with suitable *standard solutions* prepared by diminishing the sulfanilamide concentration and replacing the vitamin solution by *10% acetic acid* containing no vitamin.

Ballentine Iodate Volumetric Method for Citrus Fruit Juices.[45] For the purpose of avoiding the errors due to the changes in the solutions used in iodine titration and the increase in the volumetric error due to double back-titration by the Stevens method, Ballentine (Princeton University) adopted direct titration with iodate in acid-iodide.

REAGENTS. *Potassium Iodide Solution,* 10%. Prepare daily.

Standard Potassium Iodate Solution. Prepare a 0.1 *N* (0.0167 *M*) stock solution by dissolving 3.567 g. of KIO_3 in water and diluting to 1 liter. Dilute to 0.01 *N* as needed.

Starch Indicator. Prepare daily by dissolving 1 g. of Lintner's soluble starch in a solution of 2 g. of KI in 100 ml. of water.

PROCESS. To 5 ml. of the citrus fruit juice, add 1 ml. of *10% potassium iodide solution* and 2 ml. of *2 N sulfuric acid.* Titrate to near the end-point with *0.01 N potassium iodate solution*, then add the *starch indicator* and complete the titration, adding the standard solution dropwise.

CALCULATION. Use the equation: 1 ml. of 0.01 *N* potassium iodate = 0.88 mg. of ascorbic acid.

Ramsey and Colichman Indophenol-Iodate Potentiometric Method.[46] The method, as announced from the University of California at Los Angeles, depends upon a stable potassium iodate solution as the only standard oxidant and, at the same time, employs the specific oxidation by 2,6-dichlorophenol-indophenol.

APPARATUS. *Potentiometer*, ordinary student form equipped with a reflecting wall-type galvanometer with lamp and scale at-

tachment (sensitivity 0.014 microampere; *Agar Bridge*, saturated with potassium chloride, connecting the 200-ml. lipless titration beaker with a saturated calomel half-cell; *Platinum Electrode*, completing the circuit with the potentiometer setup; *Mercury-Sealed Stirrer* for slow uniform stirring.

Burets, 10-ml. graduated to 0.05 ml. for all iodate titrations.

Arranged in series, one source of oxygen-free nitrogen serves to blanket the solution during the preliminary steps and the titration, as well as to force the juice into the pipet.

REAGENT. *Indophenol Reagent.* 2,6-Dichlorophenol-indophenol (Eastman No. P3463) solution, 0.5 to 1 mg. per ml.

PROCESS. (*a*) *Titration with Indophenol.* Place 10 ml. of the citrus juice in the titration beaker together with sufficient *2 N hydrochloric acid* and water to make the initial resulting solution of about 100 ml. between 0.1 and 0.5 *N*. Add the *dye solution* until 1 drop remains in the oxidized form, then add approximately 10 ml. of *0.1 N potassium iodide solution* and complete the determination with *standard 0.01 N potassium iodate solution* added dropwise, allowing at least 5 seconds between the drops.

(*b*) *Titration without Indophenol.* Titrate another 10-ml. portion of the juice with *standard iodate* alone at approximately the same acidity and iodide concentration, but at any convenient rate.

FIBER

See Part I, C2*e*.

TOTAL ASH, INSOLUBLE ASH, ALKALINITY OF ASH

See Part I, C2*f*.

METALS

See Part I, C8*b*.

It is rarely true that metallic salts are present through intent. Small amounts are dissolved from storage tanks and other manufacturing apparatus and considerable amounts may be due to the incomplete removal of spray residues.

Cobaltinitrite Gravimetric Method. As tentatively adopted[47] for fruits and fruit products, but doubtless applicable to other foods, the method is as follows.

REAGENTS. *Trisodium Cobaltinitrite Solution.* Just before use, dissolve 20 g. of $Na_3Co(NO_2)_6$ in water and dilute to 100 ml.

Wash Solution. Saturate 0.01 *N* HNO_3 with dipotassium monosodium cobaltinitrite, $K_2NaCo(NO_2)_6 \cdot H_2O$.

PROCESS. *Solution.* Add to the ash of the sample *1.0 N nitric acid* to faint acid reaction, then 2 ml. more so as to give an excess of 2 ml. in the aliquot. Rinse into a 25-ml. volumetric flask, make up to volume, mix, allow to stand at least 1 hour, and filter. Pipet 10 or 20 ml., containing 3 to 25 mg. of K_2O, adjust to 20 ml. if necessary, and cool to 20°.

Precipitation. Pipet into the aliquot with stirring 10 ml. of the *trisodium cobaltinitrite solution* at 20° dropwise for 3 to 18 mg. of K_2O (preserves) or in a steady stream (lasting 20 to 22 seconds) for 18 to 35 mg. (most fruits). Let stand 2 hours at 20° under a bell-jar. Collect the precipitate of cobaltinitrite double salt in a tared sintered glass crucible, transferring with the *wash solution*, then wash at least nine times with 4-ml. portions of the *wash solution*, once with 2 ml. of *0.01 N nitric acid*, and five times with 2-ml. portions of *ethanol*, releasing the vacuum after each addition. Aspirate until dry, then dry at 100° for 1 hour, cool in the desiccator, and weigh.

CALCULATION. Obtain the milligrams of K_2O per 100 g. of the sample (*K*) by the formula

$$K = \frac{P \times 0.20738 \times 100}{A}$$

in which P is the weight of the precipitate in milligrams and A is the grams of sample in the aliquot.

PHOSPHORUS

Zinzadze Molybdenum Blue Colorimetric Method. See Part I, C8*a*.

NOTE. Gerritz [48] measures into a Kjeldahl flask 25 ml. of fruit juices and 50 ml. or more of the prepared solution of jams and jellies and proceeds as follows. Add 15 ml. of *nitric acid*, 5 ml. of *sulfuric acid*, a few *glass beads*, and small pieces of broken *porcelain*. Heat the flask on a hole in an asbestos plate smaller than the periphery of the liquid and boil to the appearance of copious fumes. If a black foam, due to charring, appears, add one or more small portions of *nitric acid*, again boil to fumes, then add 0.5 g. of *60% perchloric acid* quickly, and digest, with further additions of *perchloric acid* if necessary, until the solution is colorless or yellowish. Cool, make up to the mark in a 100-ml. volumetric flask, remove a 20-ml. aliquot, and proceed as directed by Zinzadze.

Schricker and Dawson Modification.[49] In the paper presented by Schricker and Dawson at the same time as one by Gerritz, noted above, an improved formula for the preparation of the molybdenum blue reagent is described which Gerritz found more satisfactory than the original Zinzadze formula.

APPARATUS. *Photometer*, with Jena 0–2 neutral wedge, 1-in. cell, and filter No. 66 (4.5 mm. Corning dark pyrometer red No. 241); or B & L Smoke C glass wedge and filter No. 65 (same as 66 plus 0.5 mm. of Jena BG18).

REAGENTS. *Molybdenum Blue Reagent.* Dissolve by heating with gentle mixing in a 500-ml. Kjeldahl flask 9.78 g. of 99.5 to 100% MoO_3 in 150 ml. of 36 N H_2SO_4. After cooling to 150°, slide down into the flask a small watch-glass containing 0.440 g. of very finely powdered metallic molybdenum (99.5 to 100%), maintaining the temperature at 140 to 150°, and mix vigorously until the molybdenum is practically dissolved. Cool, transfer to a 250-ml. volumetric flask, rinsing with H_2SO_4, then fill to the mark with H_2SO_4, and mix. Test the normality, which should be 0.11 N, by titrating 10 ml. of the reagent, diluted with water, with 0.1 N $KMnO_4$ solution to a pink color that persists for 1 minute. If less than 0.109 N, add a calculated amount of molybdenum and dissolve as before. Store the deep green solution in glass bottles, avoiding contamination.

For use within 8 to 10 hours, deliver from a wetted pipet 10 ml. of the reagent into 60 ml. of water in a 100-ml. flask, rinsing with water, mix, cool, dilute to the mark, and mix.

Standard Phosphate Solution. Dissolve 0.1917 g. of pure dry KH_2PO_4 in 200 ml. of water, add 10 ml. of 1.0 N H_2SO_4 and 6 drops of 0.1 N $KMnO_4$ solution, then dilute to exactly 2 liters.

PROCESS. *Solution.* Place in a 500-ml. volumetric flask 25 to 50 ml. of a solution, containing 0.5 to 2.5 mg. of phosphoric acid derived from 3.75 to 4.5 g. of fruit, the larger volume being of solutions of jellies or extracts of jams, the smaller volume of fruit juices. Add 5 ml. of *sulfuric acid*, 10 ml. of *nitric acid*, and 5 or 6 *glass beads*, then boil until the solution darkens, but avoiding excessive charring. Add 5 ml. of *nitric acid* and again boil until a slight darkening appears or sulfur trioxide fumes are evolved from an amber solution. (For jams and jellies, three or four additions may be necessary.) Finally add to the hot solution 0.5 ml. of *60% perchloric acid* and continue the fuming for a few minutes.

Caution! To avoid an explosion, never add more than 0.5 ml. of the perchloric acid and then only after practically all the organic matter has been destroyed by nitric acid (observing the precautions for the use of perchloric acid).

Cool the water-white or slightly green-yellow solution, add cautiously 50 ml. of water, boil to fumes to remove nitric acid, cool,

add 25 ml. of water, transfer to a 100-ml. volumetric flask, mix, cool, make up to the mark, and again mix thoroughly.

Color Formation. Pipet 20 ml. of the solution of the unknown and 0, 2, 4, 6, 8, 10, and 12 ml. of the *standard phosphate solution* into 100-ml. volumetric flasks with marks at 70 ml., add to the unknown 20 to 25 ml. of water, add to the standards 30 ml. of *1.0 N sulfuric acid*, and add to all the flasks 3 drops of *0.2% sodium alizarin sulfonate solution*, then exactly 10 ml. of *3.6 N sodium hydroxide solution*. Adjust by *normal acid* or *alkali* so that 1 drop of the acid just produces a yellow color. Dilute to 70 ml., swirl, heat in a boiling water bath until the full temperature is reached, then pipet exactly 10 ml. of *dilute molybdenum blue reagent* directly into the solution, swirl, and continue the heating exactly 20 minutes longer. Cool rapidly, dilute to volume, and mix. Preserve uniformity of temperature.

Photometer Reading. Determine the color values in a 1-inch cell of a neutral wedge photometer, with No. 66 (4.5 mm. Corning dark pyrometer red No. 241) filter, or the B & L Smoke C glass wedge instrument, using filter No. 65.

CALCULATION. Make a large scale graph of the standards, plotting milligrams of phosphoric acid as abscissas against photometer readings as ordinates, or calculate the equation of the line as recommended by Klein and Vorhes.[50]

BROMINE

Baughman and Skinner Chromic Acid Iodometric Method.[51] As described by Baughman and Skinner (U. S. Bureau of Chemistry), the method was designed for the determination of bromine in mineral waters and brines. The action of chromic anhydride is represented by the equation

$$2CrO_3 + 6HBr = \quad 3H_2O + 3Br_2$$

Dudley Modification.[52] The increase in the use of methyl bromide for the fumigation of fruits and vegetables in controlling insect pests led to the application, by Dudley (National Institute of Health) of the Baughman and Skinner method for the quantitative determination of bromine in the fumigated product on the market.

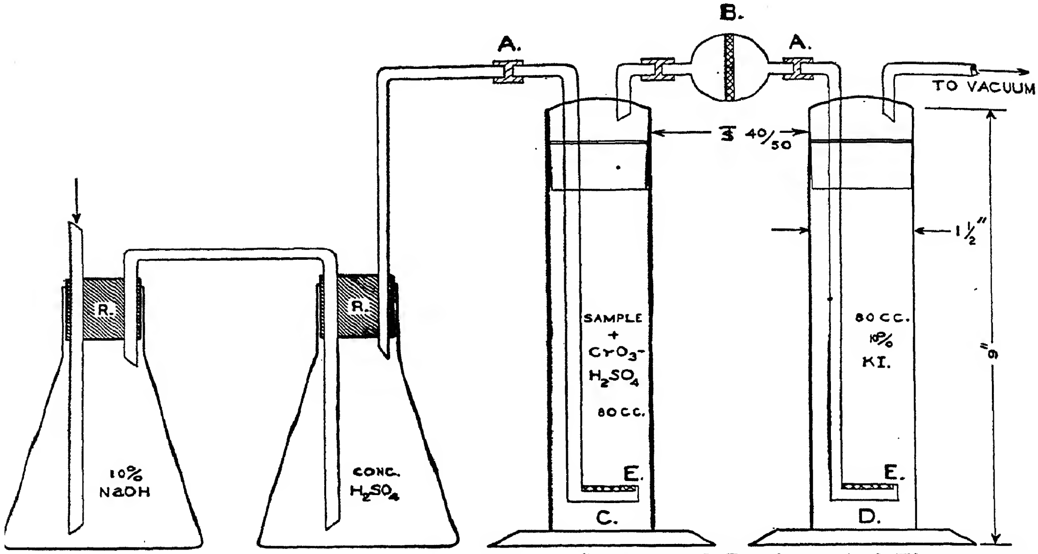
APPARATUS. *Bromine Aspiration Train* (Fig. 133).

REAGENT. *Chromic Acid Reagent*. Combine 1600 ml. of water, 200 g. of Cr_2O_3, and 600 ml. of H_2SO_4.

PROCESS. *Incineration and Extraction*. Cover 50 to 100 g. of the fruit or vegetable in a 10-ml. Pyrex or porcelain evaporating dish with about 100 ml. of *10% ethanolic potassium hydroxide solution* and let stand overnight at room temperature. Evaporate the ethanol, dry thoroughly on a hot plate, and reduce to ash in a muffle furnace at 500°. Extract with two 50-ml. portions of hot water and filter. Return the residue with filter paper to the dish, dry, and burn the charcoal, then take up in two 50-ml. portions of hot water, and filter as before. Repeat these operations a third time, taking up the ash of the third ignition in *dilute sulfuric acid* in slight excess over the alkali and carbonates, leaving the solution slightly acid as tested by an outside indicator. Filter and wash with cold water, then combine all three extracts. If the combined solution is not strongly alkaline, add a pellet of *potassium hydroxide*. Evaporate to dryness on a steam bath, taking care to avoid spattering. Take up the residue in 25 ml. of *32% by volume sulfuric acid*.

Aspiration. Transfer to bottle *C*, rinsing with 25 to 30 ml. of the *acid* delivered from a wash bottle and add 25 ml. of *chromic acid reagent*. In bottle *D*, place 80 ml. of *10% potassium iodide solution* (prepared daily), connect the train, and aspirate 20 minutes at 500 to 800 ml. per minute.

Titration. Immediately titrate the liber-



Courtesy of Ind. Eng. Chem., Anal. Ed. 1939, **11**, 259

FIG. 133. Dudley Bromine Aspiration Train.

ated iodine with *standard 0.01 N thiosulfate solution*, using *starch solution indicator*.

Dextrin

A.O.A.C. Tentative Yeast Incubation Polarimetric Method.[53] PROCESS. Dissolve 10 g. of the sample in water, add 20 mg. of *potassium fluoride* and one-quarter of a cake of *compressed yeast*. Digest below 25° for 2 to 3 hours to prevent excessive foaming, then incubate at 27 to 30° for 5 days. Defecate with *lead subacetate solution* and *alumina cream* (Part II, E3), make up to 100 ml., and polarize in a 200-ml. tube.

If the jelly is pure, the plus or minus rotation will not exceed a few tenths of a degree; otherwise, calculate as follows.

CALCULATION. Obtain the percentage of dextrin (D) as follows:

$$D = S \times 0.8755 \quad \text{or} \quad D = \frac{C \times 100}{198 \times L \times W}$$

in which S is degrees Ventzke, C is circular degrees, L is length of tube in decimeters, and W is the weight of the sample in 1 ml.

Starch

A.O.A.C. Official Test.[53] PROCESS. Prepare a 10% solution of the sample, heat nearly to boiling, add several milliliters of *10% sulfuric acid*, followed by small crystals of *potassium permanganate* until the solution is decolorized, cool, and add *iodine in potassium iodide solution*. Ignore a slight blue color that may be due to starch in apple or other jelly made from immature fruit.

Gelatin

Bömer Ethanol Precipitate Nitrogen Test.[54] Precipitate a concentrated solution of the sample with 10 volumes of *ethanol*, filter, dry the precipitate, and determine the nitrogen.

Compare the result with the percentage of nitrogen in gelatin (about 18) and in the precipitate obtained from a pure jelly.

Agar-Agar

The two following tests are A.O.A.C. Tentative Methods.

Marpmann Diatom Test.[55] Boil a portion of the sample with 5% *sulfuric acid* and a few crystals of *potassium permanganate.* Allow to settle, decant, transfer adhering diatoms to a slide with 2 to 3 drops of ethanol, and examine under the microscope.

Härtel and Sölling Precipitation Test.[56] Mix well 30 g. of the sample with 270 ml. of hot water, heat to boiling, and boil 2 to 3 minutes. Filter while hot and allow to stand at room temperature for 24 hours. If a precipitate forms, filter, wash with cold water, dissolve in a small amount of boiling water, and let cool. In the presence of 0.1 to 0.2% of agar-agar, a stiff jelly is formed.

Gelatin and Agar-Agar

Desmoulière Tests.[57] Process. *Ethanol Precipitation.* Dissolve 20 g. of the sample, in a little hot water, cool, add 100 ml. of *ethanol,* decant the liquid, and divide the precipitate into several parts.

(1) *Tannin and Picric Acid Precipitation.* Dissolve one portion of the precipitate in water. To part of the solution add *tannin,* to another part *picric acid solution.* Gelatin forms a precipitate with both reagents.

(2) Heat a portion of the precipitate with *calcium oxide.* Ammonia is split off from gelatin.

(3) Dissolve a third portion in hot water, add *lime water* to alkaline reaction, boil 2 to 3 minutes, and filter through fine linen. Neutralize the filtrate with *oxalic acid,* concentrate on the water bath, add *formaldehyde,* and evaporate to dryness. Boil the residue for a few minutes with water, filter through a hot water funnel, concentrate the filtrate to 7 to 8 ml., and transfer to a test tube. Agar-agar is indicated by the formation of a stiff jelly. Omit the formaldehyde treatment if gelatin is absent.

Artificial Colors

See Part I, C12.

Chemical Preservatives

See Part I, C13.

3.

Definition. *Cider vinegar* is the product of the successive alcoholic and acetous fermentation of apple juice. The corresponding product made from wine is *wine vinegar;* from malt or malted cereal infusion, *malt vinegar;* from dilute sugar solution, molasses, or sirup, *sugar vinegar;* from glucose or starch solution, *glucose vinegar;* and from any alcoholic distillate, *distilled vinegar.* The addition of pyroligneous acid (wood vinegar) is interdicted.

Standards. In the United States only cider vinegar can be sold as vinegar without indicating the source. In all types of vinegar 4% of acetic acid is required. Formerly 2.00 and later 1.60% of solids were the minimum legal limits for cider vinegar.

Products of Fermentation. Alcoholic and acetous fermentations take place in two stages. In the first, sugars of the hexose type, whether existing as such at the start or formed by enzymic hydrolysis, are converted through the agency of zymase produced by one of many species of yeast (*Saccharomyces*) into ethanol through anaerobic fermentation as follows:

$$\underset{\text{Hexose}}{C_6H_{12}O_6} = \underset{\text{Ethanol}}{2C_2H_5 \cdot O}$$

In the second stage the bacterium *Mycoderma aceti* (Fig. 134) brings about aerobic fermentation, on exposure to air in casks or more rapidly while passing through beech shavings, as represented by the reaction

$$\underset{\text{Ethanol}}{C_2H_6O} + 2O = \underset{\text{Acetic acid}}{C_2H_4O_2} + H_2O$$

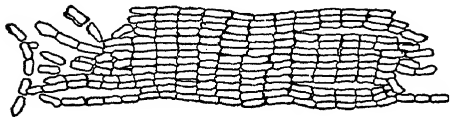


FIG. 134. Vinegar Bacteria, *Mycoderma aceti.* (Fischer.)

This reaction is really a combination of two reactions:

$$\underset{\text{Ethanol}}{C_2H_6O} + O = \underset{\text{Acetaldehyde}}{C_2H_4O} + H_2O$$

$$\underset{\text{Acetaldehyde}}{C_2H_4O} + O = \underset{\text{Acetic acid}}{C_2H_4O_2}$$

In the alcoholic fermentation, one-third of the carbon and two-thirds of the oxygen are lost as carbon dioxide; in the acetic fermentation, the oxygen is doubled and one-third of the hydrogen is lost.

None of the reactions is complete, hence a small amount of sugar escapes the first fermentation and a small amount of ethanol the second fermentation.

Other minor constituents of the fruit juice or fermenting liquid, as well as products of side reactions, are present, some of which are as follows.

Constituent Acids. In addition to *acetic acid,* cider vinegar contains *malic acid* and wine vinegar *tartaric acid,* free or combined. Crawford [58] notes that cider vinegar contains less than 0.007% of *formic acid* or a substance that responds to the test, whereas diluted pyroligneous acid contains large amounts. Rowat [59] found no formic acid in malt vinegar, hence the test is of value in detecting dilute pyroligneous acid. *Lactic Acid,* according to Hartmann and Tolman,[60] is a decomposition product of malic acid.

Glycerol and some other minor constituents are products of alcoholic fermentation. Anderson [61] detected *furfural* in 19 out of 28 vinegars known to be pure and concludes that its presence cannot be taken as evidence of the addition of pyroligneous acid or caramel.

Protein. Remington [62] suggests determination of protein as a means of distinguishing malt or sugar vinegars with high content or distilled vinegar with low content from cider vinegar.

Mineral Constituents. None of these is lost by the fermentations, although deposits may form in the casks.

Kind of Vinegar	No. of Samples	Solids	Acids Calc. as Acetic	Acids Other Than Acetic	Sugars	Ash	P_2O_5
		%	%	%	%	%	%
Cider (Lythgoe)	22	2.49	4.84	0.11 *	0.25	0.34	0.035
Wine (König)	17	1.89	5.57	0.13 †	0.35	0.27	0.053
Malt (Hehner)	7	2.70	4.23	....	....	0.34	0.105
Distilled (Paris Municipal Lab.)	12	0.35	6.34	....	trace	0.04	.....

* Largely malic, free and combined.

† Largely tartaric, free and combined.

Ethers contribute flavor and *natural colors* please the eye and contribute marks of genuineness.

Acetylmethylcarbinol. Farnsteiner,[63] Browne,[64] and Pastureau [65] found acetylmethylcarbinol in abnormal vinegar; Balcom [66] found it in normal vinegar where it constitutes the principal amount of the volatile reducing matter.

Composition. The table above gives the average composition of cider, wine, malt, and distilled vinegar.

Analysis of Vinegar. The following methods, except otherwise credited, are essentially official methods of the Association of Official Agricultural Chemists.[67]

Sample

Filter if turbid. Make all determinations on portions accurately measured with a pipet. Calculate results as grams per 100 ml.

Specific Gravity

Determine at 20° in a pycnometer or with an accurate hydrometer, both calibrated with water at 20°.

Solids

Evaporate 10 ml. of the sample, in a tared flat-bottom dish 5 cm. in diameter, to dryness or a sirup, continuing the drying in a boiling water oven for 2.5 hours. Cool in a desiccator and weigh.

Calculate results as grams per 100 ml.

Total Acids

Pipet 10 ml. of the sample into a beaker, dilute, and titrate with *0.1 N sodium hydroxide solution*, using *phenolphthalein indicator*.

If the color of wine or other vinegar interfers with observing the color changes, follow the procedure given under Red Wine. Adjustment of the dilution or selection of a suitable light will usually suffice. If not, use delicate *litmus paper* dipped in the liquor or

Calculate by the factor 0.006 as grams of acetic acid per 100 ml.

Volatile Acids

The distillation method given under Wine is not well adapted for vinegar, owing to the low volatility of acetic acid, hence in the Official Method the volatile acids are obtained by difference; the non-volatile acids are subtracted from the total acids, both calculated as grams of acetic acid per 100 ml.

Non-Volatile Acids

Evaporate 10 ml. of the sample just to dryness, dilute with 10 ml. of water, evaporate as before, repeating at least five times to remove the acetic acid. Cool, dilute to 200 ml. with recently boiled water and titrate with *0.1 N sodium hydroxide solution*, using *phenolphthalein indicator*.

Calculate as grams of acetic acid per 100 ml.

Formic Acid

See also Part I, C6*b*.

Fincke Mercurous Chloride Gravimetric Method.[68] Apparatus. *Steam Distillation Assembly.* See Part I, C6*b*, Fincke Method.

Process. *Distillation.* Pipet 100 ml. of the sample into the distilling flask, add 0.5 g. of *tartaric acid*, and connect with the absorption flask containing 15 g. of *calcium carbonate* and 100 ml. of water. Distil in a current of steam, heating both flasks so that the volume of the sample is reduced to at least half, but that of the liquid in the absorption flask remains constant. Acetic and formic acids are trapped in the absorption flask, whereas aldehydes and other volatile constituents pass on into the distillate. When 1 to 1.5 liters are collected, decant the solution in the absorption flask onto a filter.

wash by decantation, then on the paper, with hot water, leaving the bulk of the calcium carbonate in the flask.

Mercurous Chloride Precipitation. To the filtrate, add 2.5 g. of *sodium chloride*, 10 ml. of *10% mercuric chloride solution*, or a volume equivalent to at least 15 times the weight of formic acid, and heat in a boiling water bath for 2 hours. Collect the precipitated mercurous chloride on a tared Gooch crucible, wash thoroughly with water, then with *ethanol* and *ether*. Dry for 1 hour in a boiling water oven, cool in a desiccator, and weigh.

CALCULATION. Multiply the weight of mercurous chloride by the factor 0.0975, thus obtaining the weight of formic acid per 100 ml.

MINERAL ACIDS

No case of substituting dilute mineral acid wholly or in part for vinegar has ever come to the writers' notice, although a well-known scientist once confessed that he had been a party to substituting dilute sulfuric acid for acid phosphate at a soda counter.

Since all the methods originated in Europe where cider vinegar is unusual, they should be tested on samples of known purity and on mixtures with mineral acids.

Hilger Methyl Violet Method.[69] **A. Qualitative.** Dilute 10 ml. of the vinegar with an equal volume of water, or to an acidity of about 2% calculated as acetic acid, decolorizing with *bone black* if necessary, and add 2 to 5 drops of a *0.1% aqueous methyl violet solution*. If a blue or green color appears, the presence of mineral acids is indicated.

B. Quantitative.[70] Neutralize 20 ml. of the sample with *1.0 N sodium hydroxide solution*, using *litmus paper* as indicator, evaporate to about 2 ml., then dilute to about 4 ml., add a few drops of *methyl violet solution*, heat nearly to boiling, and titrate with *1.0 N acid* until the color changes from red to blue. Deduct the milliliters of acid required for the titration from the milliliters of *1.0 N* acid added at the start and calculate the remainder as mineral acids equivalent to grams of acetic acid per 100 ml. by the factor 0.06.

Conduct blank tests on vinegar of known purity and test the method on mineral acids of different dilutions.

Schidrowitz Methyl Orange Test.[71] Dilute 10 ml. of the sample with 10.5 ml. of *ethanol*. Add a few drops of *methyl orange solution*. A red-yellow color indicates mineral acids, a yellow color pure vinegar.

Richardson and Bowen Volumetric Method.[72] This method, a modification of the Hehner method,[73] has in turn been modified by Beythien [74] to overcome the disturbing influence of phosphoric acid. The principles on which it depends are that monosodium phosphate reacts neutral with methyl orange and disodium phosphate with phenolphthalein.

PROCESS. Evaporate 25 ml. of the sample with 25 ml. of *standard 0.1 N sodium hydroxide solution*, ignite below redness, and boil the cooled ash with 5 ml. of *neutral hydrogen peroxide* to avoid the later evolution of hydrogen sulfide. Boil the residue with 50 ml. of *standard 0.1 N sulfuric acid*, filter, wash, add *methyl orange* or *litmus*, and titrate with the *0.1 N alkali*; then add *phenolphthalein* and continue the titration to the end-point with that indicator.

CALCULATION. Add to the number of milliliters required for the methyl orange titration, double the additional number of milliliters required for the phenolphthalein titration and subtract the sum from 50, then subtract the difference in turn from 25, the number of milliliters of 0.1 *N* sodium hydroxide solution originally added. The latter remainder multiplied by 4 gives the mineral acid content in terms of 0.1 *N* acid per 100 ml.

REDUCING MATTER

In the official examination of vinegar as to its source, determination of reducing matter

furnishes evidence. The following methods are official.

Total Reducing Matter. PROCESS. Pipet 25 ml. of the sample into a 50-ml. volumetric flask, nearly neutralize with *1 + 1 sodium hydroxide solution*, dilute to the mark, and mix. Determine the reducing power of 40 ml. of the solution by the Munson and Walker Method (Part I, C6*a*, under Reducing Sugars).

CALCULATION. Express results as grams of invert sugar per 100 ml.

NOTE. Grab[75] demonstrated that uncolored cider vinegar does not need clarification or neutralization.

Total Reducing Matter after Inversion. Pipet 25 ml. of the sample into a 50-ml. volumetric flask, invert with 5 ml. of *hydrocloric acid*, neutralize, and determine the reducing power as above.

Non-Volatile Reducing Matter. Evaporate 50 ml. of the sample to a sirupy consistency, dilute with 10 ml. of water, and again evaporate, repeating both operations. Rinse with 50 ml. of warm water into a 100-ml. flask. If the determination of reducing matter before and after inversion shows the presence of sucrose, invert with 10 ml. of *hydrochloric acid*, nearly neutralize with *1 + 1 sodium hydroxide solution* and dilute to the mark. In the absence of sucrose, dilute to the mark directly without inversion. Determine the reducing power in an aliquot by one of the methods given in Part I, C6*a*, under Reducing Sugars.

Calculate the result as invert sugar (cider and wine vinegar) or dextrose (malt vinegar).

Volatile Reducing Matter. Deduct the percentage of non-volatile reducing matter (sugars) obtained by the preceding method from either the percentage of total reducing matter before inversion (sucrose absent) or the total reducing matter after inversion (sucrose present), thus obtaining the reducing matter (acetylmethylcarbinol) in terms of invert sugar.

NOTE. Grab[76] found that 6 samples of cider vinegar, with a total reducing matter ranging from 0.61 to 1.26, showed only 0.08 to 0.16% after evaporation.

Bender Method.[77] Bender determines pentosans as a means of detecting vinegar made from decomposed pomace or waste apples.

PROCESS. Pipet 100 ml. of the sample into a flask, add 43 ml. of *hydrochloric acid*, and proceed as described in Part I, C6*a*.

ALCOHOL PRECIPITATE

Bender Method. For the same reason as given in the preceding section, Bender recommends the determination of alcohol precipitate, the method being as follows.

PROCESS. Evaporate 100 ml. of the sample to 15 ml. or, if the sugar content is high, to 20 ml., thus avoiding a gummy or stringy precipitate. Add to the residue slowly with stirring 200 ml. of *95% by volume ethanol* and allow to stand overnight. Complete the determination as directed for the determination of pectin above.

ETHANOL

Distillation Method. Pipet 100 ml. of the sample into a distillation flask, neutralize with *sodium hydroxide solution*, leaving the solution faintly alkaline, add a piece of *paraffin* the size of a pea or a little *tannic acid*, and distil until nearly 50 ml. have passed over. Make up the distillate to 50 ml. at 20° and determine the specific gravity at 20°.

Martini and Nourrisson Dichromate Iodometric Method.[78] The method, which originated at the Oenological Station at Dijon, is designed to determine very small amounts of ethanol, especially in vinegar. The ethanol is distilled into an oxidizing mixture with the formation of acetaldehyde and the excess of

dichromate is determined by iodometric titration. The reactions involved are:

$$2K_2Cr_2O_7 + 8H_2SO_4 + 3CH_6O \rightarrow$$
$$2Cr_2(SO_4)_3 + 2K_2SO_4 + 3C_2H_4O_2 + 11H_2O$$
$$K_2Cr_2O_7 + 6KI + 7H_2SO_4 \rightarrow$$
$$Cr_2(SO_4)_3 + 4K_2SO_4 + 7H_2O + 6I$$
$$+ 2I \rightarrow Na_2S_4O_6 + 2NaI$$

APPARATUS. *Martin Distillation Assembly* consisting of (1) a round-bottom flask provided with a perforated rubber stopper carrying a long glass tube bent near the stopper at an angle of about 30° and (2) a long glass tube, closed at the end, of about 75 ml. capacity, serving as a receiver. No condenser is necessary.

PROCESS. *Oxidation and Distillation.* Dilute the vinegar to 5 or 10 times its volume with water and pipet 10 ml. of the liquid into the distilling flask. Place in the receiving tube 20 ml. of *0.01667 N potassium dichromate solution* and 10 ml. of *sulfuric acid* free from sulfurous acid; then while the dichromate-acid mixture is still hot distil the contents of the flask. Observe the color of the oxidizing mixture as the distillation progresses. If it is bright green with a blue tint, the amount of dichromate is insufficient to oxidize all the ethanol and the determination must be repeated, a charge of greater dilution of the sample being used.

Titration. Transfer the liquid in the receiving tube to a 500-ml. Erlenmeyer flask, dilute to 250 ml., add 10 ml. of *5% potassium iodide solution* (free from iodate), and titrate with *0.1 N thiosulfate solution* until the green-yellow color changes to green-blue. Addition of starch indicator is unnecessary.

CALCULATION. By using a five-fold dilution of the unknown, the percentage of ethanol (E) is obtained by the following formula:

$$E = (20 - N) \times 0.00144 \times 50$$

in which N is the number of milliliters of thiosulfate solution used in the titration. If the dilution is ten-fold, substitute 100 for 50 in the equation.

GLYCEROL

Hehner Dichromate Oxidation Volumetric Method.[79] The reaction involved follows:

$$3C_3H_8O_3 + 7K_2CrO_7 + 28H_2SO_4$$
$$7K_2SO_4 + 7Cr_2(SO_4)_3 + 9CO_2 \cdot 40H_2O$$

Richardson and Jaffé[80] shortened the time required for the reaction by increasing the strength of the dichromate.

Ross Modification.[81] The modification has been studied, improved, and officially adopted by the Association.[82]

REAGENTS. *Potassium Dichromate Solution.* Strong: Dissolve 74.56 g. of dry, recrystallized $K_2Cr_2O_7$ in water, add 150 ml. of H_2SO_4, cool, and make up to 1 liter; 1 ml. = 0.01 g. of glycerol. Dilute: Make 25 ml. of the preceding up to 500 ml. Make all measurements at 20°.

Ferrous Ammonium Sulfate Solution. Dissolve 30 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in water, add 50 ml. of H_2SO_4, cool, and dilute to 1 liter at room temperature.

PROCESS. *Evaporation.* Pipet 100 ml. of the sample into a flat-bottom dish and remove acetic acid by evaporation to about 5 ml. on a water bath kept at 85 to 90°, then dilute with 20 ml. of water and again evaporate to 5 ml. Add 5 ml. of fine *quartz sand* and 15 ml. of the freshly prepared *milk of lime* and evaporate, with frequent stirring, nearly to dryness.

Ethanol Extraction. Make into a homogeneous paste with 5 ml. of hot water, add 45 ml. of *absolute ethanol,* washing down the sides of the dish and stirring thoroughly, then heat just to boiling on a water bath kept at 85 to 90°. Filter on a pleated paper, wash twice by decantation, then on the paper with hot *90% ethanol* until the filtrate measures 150 ml.; or, better, centrifuge after each ad-

dition, wash by decantation, and complete the washing on the paper. In both cases, collect the filtrate and washings in a porcelain dish.

Ethanol-Ether Extraction. Regulate the temperature at 85 to 90°, evaporate the filtrate and washings to a sirup, mix with 10 ml. of *absolute ethanol*, and transfer to a 50-ml. glass-stoppered cylinder, rinsing with *absolute ethanol* to a total of 20 ml. Shake with 10 ml. of *absolute ether* and pour off when clear onto a filter; repeat twice and finally wash the cylinder and paper with *ethanol-ether wash liquid.* If a heavy precipitate has formed in the cylinder, centrifuge at low speed, decant the clear liquid on a paper, and wash three times by mixing well with *ethanol-ether wash liquid* (10 + 15, both absolute), centrifuging and decanting on the paper. Evaporate the filtrate and washings at 85 to 90° to about 5 ml., add successively 20, 20, and 10 ml. of water and evaporate to about 5 ml. after each, thus removing ethanol and ether.

Silver-Lead Precipitation. Transfer the concentrated liquid with hot water to a 50-ml. volumetric flask, cool, add the washed and suspended precipitate of *silver carbonate* from 0.1 g. of silver sulfate, shake occasionally, and allow to stand for 10 minutes, then add 0.5 ml. of *lead subacetate solution*, shake occasionally, and allow to stand for 10 minutes. Dilute to the mark, shake well, and filter, rejecting the first portions of the filtrate. Pipet 25 ml. of the clear filtrate into a 250-ml. glass-stoppered volumetric flask, and delead with 1 ml. of *sulfuric acid.*

Oxidation. To the glycerol in the 250-ml. flask, add 30 ml. of the *strong dichromate solution* (35 ml. for high glycerol content), measured from a pipet with the greatest accuracy, which suffices to leave about 12.5 ml. in excess, then cautiously add 24 ml. of *sulfuric acid*, rolling the flask gently. Heat in a boiling water bath for exactly 20 minutes, dilute at once, cool, make up to the mark, and reserve for the titration.

Titration. Pipet accurately 20 ml. of the *ferrous ammonium sulfate solution* into a beaker, dilute with 100 ml. of water, and titrate with the *dilute dichromate solution*, using *1% potassium ferricyanide solution* as an outside indicator on a porcelain spot-plate.

In like manner titrate 20 ml. of the *ferrous ammonium sulfate solution* against the solution of the oxidized glycerol containing the excess of strong dichromate solution.

Calculation. Obtain the grams of glycerol (G) per 100 ml. of the sample by the formula:

$$G = D - 0.01 \times 2 \times \frac{250d}{20g} = D - \frac{d}{4g}$$

in which D (usually 30) is the milliliters of strong dichromate added for oxidation, and d and g are respectively the milliliters of dilute dichromate solution and glycerol solution required in the titration.

Note. In the Official Modification, preliminary to the titration are added 20 ml. of a *retarder* (prepared by diluting 150 ml. of sirupy phosphoric acid with 600 ml. of water and 200 ml. of sulfuric acid), then 4 drops of an *inside indicator* (a solution of 1 g. of diphenylamine in 100 ml. of sulfuric acid). The titration is carried on at the usual rate until the liquid assumes a green color, then dropwise with continual stirring until the color changes from blue-gray to deep violet.

Oxidation Number; Iodine Number; Ester Number

Edwards and Nanji Method of Differentiating Genuine and Spurious Vinegar.[53] Process. A. Oxidation Number (*Milliliters of 0.01 N potassium permanganate required to oxidize 100 ml. of the sample in 30 minutes under standard conditions*).

Distillation. Mix 60 ml. of the sample with 15 ml. of water in a 400-ml. distilling flask, add a little *pumice*, and distil slowly until 60 ml. of distillate have been collected.

Oxidation. To 25 ml. of the distillate, contained in a 200-ml. glass-stoppered bottle, add 10 ml. of *1 + 3 sulfuric acid* and 10 ml. of *0.1 N potassium permanganate solution.* Let stand 30 minutes at 18°, then add 5 ml. of *10% potassium iodide solution*, and titrate the liberated iodine with *0.02 N sodium thiosulfate solution*, using *starch solution* as indicator when near the end-point.

Make a blank determination, using 25 ml. of water with the same reagents and in the same manner as in the actual analysis.

CALCULATION. Obtain the oxidation number (O) by the following formula:

$$O = 8(B - A)$$

in which B and A are the number of milliliters of 0.02 N thiosulfate solution respectively required for the blank and the actual analysis.

B. IODINE NUMBER (*Milliliters of 0.01 N iodine solution absorbed by 100 ml. of the sample under standard conditions*). Neutralize to *litmus paper* 25 ml. of the above distillate, contained in a 200-ml. glass-stoppered bottle, with *10 N potassium hydroxide solution*, add 10 ml. of *1.0 N potassium hydroxide solution* and 10 ml. of *0.1 N iodine solution*, and allow to stand in a dark place for 15 minutes, then add 10 ml. of *9 N sulfuric acid* and titrate with *0.02 N sodium thiosulfate solution.*

Make a blank determination on 25 ml. of water, as in the actual analysis.

CALCULATION. Obtain the iodine number (I) by the following formula:

$$I = 8(B - A)$$

in which B and A are the number of milliliters respectively of 0.02 N thiosulfate solution required for the blank and the actual analysis.

C. ESTER NUMBER (*Milliliters of 0.01 N potassium hydroxide solution required to saponify the esters in 100 ml. of the sample under standard conditions*). Pipet 100 ml. of the sample into a 400-ml. distillation flask, add a little pumice, distil slowly until 30 ml. of distillate are collected, and add *phenolphthalein* and *1.0 N potassium hydroxide solution* to the distillate until a pink color appears. Add *0.02 N hydrochloric acid* dropwise until the color is discharged, then add 10 ml. of *0.1 N potassium hydroxide solution*, and saponify by refluxing on the water bath for 2 hours. Cool, add more *phenolphthalein*, and titrate with *0.02 N hydrochloric acid.*

Make a blank determination as follows. Reflux 30 ml. of water with 10 ml. of *0.1 N potassium hydroxide solution* and titrate with *0.02 N hydrochloric acid* as in the actual analysis.

CALCULATION. Obtain the ester number (E) by the following formula:

$$E = 2(B - A)$$

in which B and A are the number of milliliters of 0.02 N acid respectively required by the blank and the actual analysis.

INTERPRETATION. *Oxidation.* Spirit vinegar has a markedly higher number than artificial vinegar, including wood vinegar.

Iodine Number. Diluted acetic acid has a rather low number, pure spirit vinegar a somewhat higher, and wood vinegar often a very high number.

Ester Number. Artificial vinegar has an extremely low number; unfortunately pure spirit vinegar, after long storage, also has a low number.

COLOR

Bender Method for Total Color.[84] Compare in a 0.5-in. cell of the Lovibond tintometer with slides of the brewer's scale.

Bender Method for Color Removed by Fuller's Earth.[84] Shake 50 ml. of the sample with 25 g. of *fuller's earth* at intervals for 30 minutes and filter through a fluted paper. Dilute to 50 ml. equal volumes of the treated and untreated samples in colorimetric tubes and compare. Standardize the fuller's earth against samples colored with caramel and uncolored samples. Express results in terms of per cent of color removed.

Caramel

Lichthardt Tannic-Sulfuric Acid Test. This test, designed for vanilla extract (Part II, J3), has been adapted by Cook and Miller. [85] and approved by Gulick [86] for the detection of caramel in vinegar.

Reagent. Dissolve 9.8 g. of tannic acid in about 300 ml. of water, add 4 ml. of H_2SO_4, and dilute to 490 ml. Just before use, mix 4 ml. of formaldehyde with 96 ml. of the stock reagent.

Process. Mix 5 ml. of the sample with 5 ml. of the *reagent*, immerse for 4 minutes in boiling water, then let stand overnight.

A brown precipitate deposited at the bottom indicates caramel. Disregard a gray precipitate.

Ash

The official A.O.A.C. method involves evaporation of 25 ml. of the sample, burning at 500 to 550°, extraction with water, filtration, washing, ignition of the filter and contents, addition of the filtrate, then evaporation and ignition as before.

Soluble and insoluble ash and the alkalinity of each are determined as directed in Part I, C2*f*.

Phosphoric Acid

See Part I, C8*a*.

4. COMMERCIAL PECTIN

The gelatinizing substance extracted from apple pomace and citrus albedo is the vegetable analogue of commercial gelatin. The product sold under the name Certo, as well as powdered pectin, is suitable for family use.

Jelly Grade

Bailey Tartaric Acid Method. The following details were furnished by H. S. Bailey, Manager of The Exchange Orange Products Company, Ontario, California. The directions assume that the approximate jelly grade is known; if not, make up a [illegible] jelly, a [illegible] a grade of 100 f[illegible] all dry commercial pe[illegible]

Apparatus. *Four-Quart* [illegible] *Saucepan* and *Spoon*.

Regulation 6 oz. Jelly [illegible] Hazel Atlas No. 85.

Reagent. *Standard d-Tartaric Acid Solution.* Dissolve 376.2 g. of $C_4H_6O_6$ in water and dilute to 1 liter.

Process. *Preparation of Mixture.* Weigh on a piece of tared paper 388 g. of *cane sugar* and in a tared scoop or beaker a quantity of the sample equal to the sum of the weight of sugar plus weight of pectin, divided by the assumed pectin grade; for 100 grade pectin this would be 3.92 g. Mix the pectin in a 150 ml.-beaker with about one-third of the weighed sugar, place in the saucepan 250 ml. of water, add the *pectin-sugar mixture*, and stir until the pectin is thoroughly dispersed. Heat quickly to boiling, add the remainder of the *sugar*, and bring to a full boil. Remove the pan and contents from the fire and weigh. If the contents weigh more than 600 g., boil off more water; if already less than 600 g., add water in excess and boil again to correct weight.

Gelatinization. Skim, pour into the jelly glasses, into each of which has previously been run from a pipet 2 ml. of *standard d-tartaric acid solution*, and stir to mix the acid thoroughly. Allow the jellies to stand at room temperature 12 to 15 hours.

Jelly Test. Invert the glass and slip the mold of jelly out onto a suitable tray by loosening the jelly from the glass with a dampened spatula. If the pectin was of the grade assumed, the jelly will be firm enough to stand up without sagging more than 23% of its original height in the glass. It will have a good resilience when gently squeezed and show when broken, after being cut part way through with a clean spatula, a [illegible] not grainy, cleavage.

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E. SACCHARINE FOODS

Chemical Control. The sugar industry is under chemical control at all stages of production and manufacture. In breeding varieties of cane and beet to secure the maximum yield of sucrose, the sugar content is determined from bottom to top of the sugar cane and from root tip to crown of the sugar beet. In everyday operation, lots of cane or beets are examined in the sugar house and processes are regulated to secure the maximum yield. The products and by-products are analyzed at the various stages of manufacture, ingenious devices being employed to facilitate the work.

In the refinery the control is a model of efficiency; a fraction of a per cent of sugar means tons in the aggregate and a small fraction of a cent in price per pound amounts to millions of dollars in a country, like our own, of high sugar consumption.

1. SUGAR CANE AND SUGAR BEET

General Composition. *Cane juice* is the saccharine liquid expressed from sugar cane after milling. When the diffusion process is employed, the true juice is diluted with water. The *bagasse* remaining after the juice is expressed still contains a small amount of sugar.

Massecuite is the product obtained by evaporating the juice to the crystallizing stage. By successive evaporation, several crops of raw sugar are separated. The mother liquor is molasses.

Raw sugar cane juice, as given by Browne and Blouin,[1] contains on the average: solids 15, sucrose 12, dextrose, levulose, and invert sugar 1.70, and ash 0.45%. Raw beet juice contains approximately: sucrose 18.5, non-sugars 9.0, and ash 2.0%.

Sample

A representative number of canes or beets from different parts of the field or the harvested crop are selected. Experience in this detail is of paramount importance. The leaves are stripped from the canes and the tops cut off from the beets. Dirt is removed and abnormal specimens that should not be included in a fair sample are rejected. Delay and consequent spoilage must be avoided. The methods, if not imitating in miniature the manufacturing process, should at least give comparable results. The stripped sugar canes are milled in laboratory apparatus, the sampling and extraction of the sugar being combined in the operation.

Although the sugar content is not uniform throughout the sugar beet, it is not necessary to pulp the whole beet, since a core removed by a boring rasp directed diagonally from the middle of one side to the edge of the crown on the opposite side is representative. The mixed pulp from a suitable number of beets is reduced by subsampling to manageable proportion and, if necessary, further pulped to insure complete extraction. Cosettes (beet slices) are sampled and subsampled in the usual manner.

Sucrose

Sugar cane is milled and extracted in laboratory apparatus fashioned in miniature on the plan of the mill equipment. Since the extraction, or heavy pressing process, is al-

most universal in the United States, not the diffusion or soaking process with nearly complete solution of the sucrose, it is important that the sucrose content of the juice, not the full content of the cane, be known.

Sucrose is determined in the juices after defecation with lead subacetate solution by direct polarization in a 400-mm. tube in the usual manner. The Brix reading in percentage of sugar furnishes information more readily, but less accurately.

Cane juice from the pipes of the sugar house is examined in the same manner as that extracted from samples of the cane. Simple though the processes may seem from a brief survey, no little experience and judgment are brought into play in mill control.

Sugar beets are treated differently in the laboratory from sugar cane. The diffusion process extracts nearly all the soluble constituents, hence the total sucrose content is significant.

The following methods, differing in the process of digestion, are of general application, since they are adapted with modification to the extraction and determination of sugar in fruits, as well as in certain vegetables (e.g., green corn, carrots, cucurbits, etc.), and even cattle foods (e.g., mangolds, ensilage, etc.). Ethanol and cold water extraction methods having been shown to yield low results, only two methods, employing hot water as a solvent, are described below.

Modified Pellet Hot Water Digestion Polarimetric Method.[2] Reagent. *Lead Subacetate Solution.* See Part II, E2, below.

Process. *Defecation.* Mix in a 201-ml. graduated flask 26 g. of the finely pulped material, add 5 to 10 ml. of *lead subacetate solution* and water sufficient to fill the flask to 160 to 170 ml., and shake.

Digestion. Heat in a bath at 80° for 30 minutes, rotating at intervals, make up to the mark with water at 80°, digest for 10 minutes longer, and cool to 20°. Make slightly acid with *acetic acid* (about 0.5 ml.), add a little *ether*, and apply suction to break the foam.

Polarization. Finally make up to the mark at 20°, mix, filter, and polarize in a 400-mm. tube, also after inversion as directed under E2 below.

Calculation. The direct reading is the percentage of crude sucrose. Calculate pure sucrose by Clerget's formula or a modification.

Modified Sachs-Le Docte Hot Water Digestion Polarimetric Method.[3] The difficulty of adjusting the frothy liquid to the mark is obviated by adding an accurately measured volume of dilute lead subacetate solution, which together with the water in the sample gives the desired volume.

Apparatus. *Nickel-Plated Sheet Iron Flask*, 11 cm. high, 6 cm. in diameter, with a neck 4 cm. in diameter.

Reagent. *Lead Subacetate Solution*, dilute. Mix 5 volumes of $Pb(C_2H_3O_2)_2 \cdot Pb(OH)_2$ (Part II, E2) with 100 parts of water.

Process. Add to 26 g. of the finely pulped sample, contained in the metal flask exactly 177 ml. of *dilute lead subacetate solution.* The moisture in 26 g. of sugar beet pulp of average composition brings the total volume of liquid up to 200 ml.

Digestion. Stopper the flask loosely and heat in a bath at 75 to 80°, tighten the stopper after 5 minutes, and continue the heating to a total of 30 minutes.

Polarization. Shake, cool to standard temperature, filter, add a drop of *acetic acid*, and polarize in a 400-mm. tube. Also polarize after inversion as directed under E2 below.

Calculation. The reading on the sugar scale is the percentage of crude sucrose. Obtain pure sucrose by Clerget's formula or a modification.

Invert Sugar

Delead the solution, prepared by one of the above methods, and determine reducing

sugars, either volumetrically or gravimetrically, as under E2 below.

Calculate the result in terms of invert sugar.

Fiber

The cellular and other substances insoluble in water without coagulation constitute the "fiber."

Wiechmann Hot Water Extraction Gravimetric Method.[4] Crush 10 to 20 kilos of the cane in a laboratory mill, express about 75% of the juice, cut the bagasse into small pieces, mix, and weigh. Extract 20 g. of a finely ground subsample, on a weighed filter, first with water below 50° to insure removal of coagulable material, then with water at about 70°, and finally with water heated nearly to boiling, until all soluble matter is removed, the whole operation requiring about 45 minutes. Dry the fiber to constant weight in a boiling water oven and calculate the percentage.

2. COMMERCIAL SUGAR (CANE AND BEET SUGAR)

Raw cane sugar, as examined by Zerban[5] at the New York Sugar Trade Laboratory from 1925 to 1929, contained on the average 95.82 to 96.86% of sucrose.

According to the U. S. Standards, white sugar, whether from sugar cane or sugar beet (granulated, loaf, cut, milled, and powdered), must contain at least 99.5% of sucrose.

The relative amounts of sugar made from sugar cane and sugar beets in the world varies from decade to decade and even from year to year. Just before World War I, as stated by Horne, the production of cane sugar was little more than 10% greater than that of beet sugar, but in the 10 years beginning with 1927 it was nearly 100% greater. In Europe practically all the sugar is produced from sugar beets, in the Gulf States of the United States and the West Indies, from sugar cane. At present sorghum is used only for sirup, although in the 1870's it bid fair to be an important source of white sugar. The Aztecs, also the patriots during the American Revolution when cut off from their West Indian supply, made sugar from corn (maize) stalks. Other species from the stalks of which sugar is produced commercially are several species of palm (nipa, date, sugar, and palmyra) and the grape vine.

Refined cane and beet sugar differ only in origin. Both consist of nearly chemically pure sucrose. The term cane sugar, applied to sucrose, is confusing; the less specific term sugar is here employed regardless of origin.

Certain grades of brown sugar made from sugar cane, once regarded as inferior, now command a higher price in retail trade because of the desirable flavor. Beet sugar is suited for human food only when refined. Raw beet sugar and molasses are not relished even by animals and the latter is largely utilized in alcohol manufacture.

Analytical Methods. Usually direct polarization supplemented in some instances by determination of solids and ash, suffices for the analysis of white sugar. High grade granulated sugar is practically a chemically pure product, deviating little from a direct polarization of 100. Tales of sand in sugar are fantastic. Never in sixty years of experience has a single authentic case of adulteration of sugar come to the writers' notice. This cannot be said, however, of maple sugar or of cane sugar by-products. Impurities in raw and the various grades of brown sugar are largely water, coloring matter, and invert sugar.

Sample

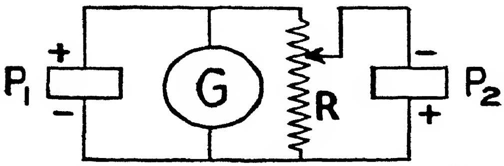
Drawing and preparing for analysis a representative sample of sugar, whether raw or refined, present no greater difficulty than is encountered in the sampling of other granular or powdered products.

If in large packages, remove cores with a sampling tube from several packages; if in small containers, use the entire contents of one or more. Mix, ring, and halve the large composite sample thus obtained,.repeating until the quantity is reduced to manageable size. Finally mix thoroughly the subsample before bottling and again mix with a spoon before weighing out portions for analysis. Excepting sugar loaves or cubes, grinding is not usually necessary, since the large quantity weighed out for determination of sucrose compensates for error due to the size of the particles.

Grading of White Sugars

Keane and Brice Photoelectric Method.[6] The method, developed in two United States Bureaus, obviates the personal equation of visual measurement.

Apparatus.[7] *Compensating Photoelectric Circuit* (Fig. 135).



Courtesy of Ind. Eng. Chem., Anal. Ed. 1937, 9, 259

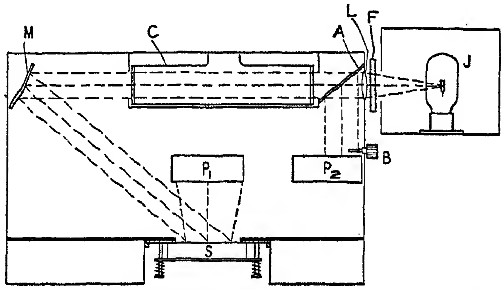
Fig. 135. Keane and Brice Photoelectric Circuit for Sugar Grading.

Photoelectric Photometer (Fig. 136). The beam from a 150-watt projection lamp (J) passes through a color filter (F). It is made parallel by a lens (L) and divided by a clear glass plate (A); 10% is reflected onto a compensating photocell (P_2) while the main beam is transmitted by the plate (A); C absorption cell; M collimating mirror; S standard white reflecting surface which may be replaced by the sample; P_1 measuring photocell.

Process. *Reflectance.* Place the granulated sugar in a metal dish 63 mm. in diameter and 16 mm. deep and carefully smooth the surface with a Petri dish having a flat, ground bottom. Insert in the proper slide against a 56-mm. opening. Calculate the error of overfilling (Δd) or underfilling ($-\Delta d$) by the formula

Since d is 55 mm., overfilling or underfilling by 0.3 mm. causes an error of $0.011R$ of about 1% of the reflectance (R).

With the standard white plate in position, adjust the galvanometer to read 0, set the potentiometer-rheostat scale at 85.6 (reflectance of standard white plate), turn on the lamp, and move the shutter (B) until the galvanometer again reads 0. Replace the standard plate by the sample and restore the



Courtesy of Ind. Eng. Chem., Anal. Ed. 1937, 9, 259

Fig. 136. Keane and Brice Photometric Apparatus for Sugar Grading.

balance by adjusting the potentiometer-rheostat. The scale then indicates the reflectance of the sample relative to magnesium oxide.

Transmittance. Dissolve 150 g. of sugar in water at room temperature and dilute to exactly 250 ml. Remove bubbles by applying a vacuum or allowing to stand several hours. Fill a 150-mm. absorption cell and allow to stand 1 minute for newly formed bubbles to rise. Fill a half-length cell with the same water for use as a reference cell. Balance the

circuit, as described for reflectance measurement, except that at the start the scale is set to read 100 with both the standard white plate and the 75-ml. cell in the light beam. After balancing the circuit, replace the water cell by the 150-mm. solution cell, then when the circuit is rebalanced read the scale showing the transmittance.

Moisture

Hard-and-fast rules for moisture determination do not always lead to agreement by different analysts with different apparatus, working in different localities or on different days with varying degrees of barometric pressure and relative humidity. Each analyst must be guided by circumstances and make comparative analyses under like conditions.

The following are A.O.A.C. Methods.

Air Oven Gravimetric Method. Dry 2 to 5 g. of the sample in a boiling water oven for 10 hours, cool, and weigh, then dry for 1-hour periods until the loss is no more than 2 mg., which for 2 g. is 0.1, for 5 g. 0.04%. If the sugar has large grains, heat at 105 to 110° to expel the last traces of occluded moisture.

Vacuum Oven Gravimetric Method. Proceed as in the direct method, using, however, a vacuum oven heated at below 70° under pressure not exceeding 50 mm. for 2 hours, and then for 1-hour periods to constant weight.

This method is used chiefly when considerable invert sugar is present.

Sucrose

Polarization Methods. By far the most important application of the polariscope is in the determination of sucrose. The planter, the manufacturer of raw sugar, and the sugar refiner value their products by the results of polariscopic analysis. Customs officials base duties and drawbacks on the percentage of sucrose in sugar and other saccharine products, as determined by polarization, and official food chemists employ polarization methods for the examination of products with respect to their compliance with regulatory statutes.

Polariscope. Instruments designed for general research and the examination of certain commercial products, notably essential oil, are constructed with a scale showing angular degree of rotation, but the forms made for the sugar chemist, the saccharimeters, have a sugar scale which shows directly percentages of sucrose in solutions containing a normal (standard) weight of the sample made up to a standard volume and examined in a standard length tube, provided other sugars or optically active substances are not also present.

The normal weight for instruments with the Ventzke scale is 26.026 g., for the U. S. Bureau of Standards scale is 26.000 g., special weights being furnished for both quantities by the instrument makers. After being dissolved in water, the solution is diluted to 100 ml. in a volumetric flask. Examination is made in a 200-mm. tube. Angular rotation degrees may be converted into Ventzke degrees by the factor 2.88542 (2.88850 U. S. Bureau of Standards scale) and readings on the Ventzke scale to angular degrees by the factor 0.34657 (0.34620 U. S. Bureau of Standards scale), sodium light (D) being used in all cases.

Conversion factors are essential when an instrument with a sugar scale is used for the examination of essential oils and other materials the polarization of which is expressed in angular degrees.

High grade granulated sugar dissolved and examined in a saccharimeter in the manner indicated shows readings varying little if any from 100. If, however, other optically active sugars, notably dextrose, levulose, invert sugar (1 + 1 dextrose and levulose), raffinose, maltose, and lactose, are present, polarization after inversion and calculation of the

tion is automatically introduced if the solution is polarized in a 220-mm. tube.

CALCULATION. Obtain the percentage of sucrose by the Clerget formula given above.

I. Browne Room Temperature Inversion Modification.[9] For the purpose of correcting the errors of the Clerget procedure, Browne, although retaining Clerget's addition of one-tenth for inversion, introduces the following simple changes in manipulation and calculation.

Inversion. Place 50 ml. of the solution used for the direct polarization in a 50- to 55-ml. volumetric flask, add 5 ml. of *hydrochloric acid,* and allow to stand overnight at a temperature not below 20°. Complete the volume to 55 ml. after gently tapping to detach air bubbles, mix, and polarize in a 200-mm. tube in the usual manner.

CALCULATION. Increase the invert polarization reading by one-tenth. Calculate the sucrose (S) by the following formula:

$$S = \frac{100(P - P')}{133.15 + 0.0673g - 0.5(t - 20)}$$

in which P and P' are the direct and invert polarization respectively, g is the grams per 100 ml. of dry substance of the original solution used for the polarization, and t is temperature.

II. Herzfeld Modification.[10] This modification of the Clerget process has been more generally used, at least in the United States, than the original method.

PROCESS. *Direct Polarization.* Proceed as in the Clerget Method.

Invert Polarization. Pipet into a 100-ml. volumetric flask 50 ml. of the clarified and filtered solution of the sample, dilute with 25 ml. of water, add 10 ml. of *hydrochloric acid,* and mix. Introduce a thermometer and heat in a water bath at 72 to 73° at such a rate that the solution in the flask reaches 69° in 2.5 to 5 minutes. Maintain at 69° for 5 minutes with occasional rotation, then remove from the bath, cool rapidly to 20°, and polarize in a 200-mm. tube.

CALCULATION. Multiply the reading by 2 to compensate for the dilution and calculate by the following formula, the significance of the letters being the same as in the Clerget formula:

$$S = \frac{100(P - P')}{132.66 - 0.5(t - 20)} = \frac{100(P - P')}{142.66 - 0.5t}$$

In the A.O.A.C. method, 143 is substituted for 142.66 which has been shown to be too low.

III. Schrefeld Modification.[11] Attention has been called above to inaccuracies of the Clerget-Herzfeld method caused by a slightly erroneous factor and the influence of the concentration of the solution. An investigation by an International Committee, interrupted by World War I, was continued by Zerban[12] in collaboration with members of the Association of Official Agricultural Chemists. After exhaustive studies, details of manipulation (essentially as proposed by Schrefeld) and formula were adopted by the Association[13] essentially as follows.

PROCESS. *Direct Polarization.* As in the Clerget Method.

Indirect Polarization. Pipet 50 ml. of the cleared solution into a 100-ml. flask, dilute with 25 ml. of water, add 10 ml. of *hydrochloric acid* (sp.gr. 1.1029 at 20°/4°). Heat in a water bath with continual agitation at 70° until a thermometer in the flask indicates 67°; this should require 2.5 to 2.75 minutes. Continue the heating 5 minutes longer, thus causing a gradual rise in temperature to about 69.5°, then plunge at once into water at 20°. When the contents of the flask reach about 35°, remove and rinse the thermometer, fill almost to the mark, and leave in the bath at 20° for at least 30 minutes longer. Finally adjust accurately to the mark, mix, and polarize at 20° in a 200-mm tube in a jacketed tube with a lateral branch.

Multiply the reading by 2. Departure from 20°, within narrow limits, is permitted, provided the same temperature is maintained for the several operations.

The inversion may also be carried out at room temperature, not below 20° for 24 hours or over 25° for 10 hours, using the formula below, except that the factor 143.2 is substituted for 143.0.

CALCULATION. Calculate by the formula

$$S = \frac{100(P - P')}{143 + 0.0676(s - 13) - 0.5t}$$

The letters P, P', and t have the same significance as in the foregoing formulas; s is grams of total solids in 100 ml. of the inverted solution read in the polariscope, as determined by multiplying the percentage by weight derived from the refractometer reading (International Commission—A.O.A.C. Table) by the specific gravity at 20° corresponding to the percentage by weight of sucrose as derived from Domke's table (E3, below).

NOTE. If much levulose is present, as in honey, fruit products, sorghum sirup, and molasses, it is stated that the above method gives erroneous results. This is true of all acid inversion methods.

IV. Saillard Equalization Modification.[14] In addition to deleading and neutralization, further equalization is attempted by adding to the solution for direct polarization an amount of *sodium chloride* equal to that formed by neutralization.

PROCESS. The details of the method, as described by Saillard, Wehrung, and Ruby,[15] are essentially as follows. Defecate 52 g. of the sample with 20 ml. of *lead subacetate solution*, make up to 200 ml., filter, and delead 100 ml. with *sulfurous* or *oxalic acid*, removing the excess with dry *calcium* or *barium carbonate*. Decolorize, if necessary, with *animal charcoal* and filter. To 50 ml. of the solution, add 4.2 g. of *sodium chloride* or 5.3 g. of *potassium chloride*, make up to 100 ml., and polarize at 20°. Invert another 50-ml. portion with 6.5 ml. of *hydrochloric acid*, neutralize the acid with *sodium* or *potassium hydroxide solution*, according as sodium or potassium chloride was added to the solution for direct polarization, make up to 100 ml., and polarize at 20°.

The method, although original and logical, does not appear to have met with favor.

V. Andrlik–Stanek Modification.[16] Instead of equalizing the conditions of the two polarizations by neutralizing the inverted solution and adding a corresponding quantity of alkali chloride to the solution for direct polarization, Andrlik and Stanek add to 50 ml. of the cleared solution 5 ml. of *hydrochloric acid* and 5 g. of *urea* and make up to 100 ml., the action of the urea being to retard inversion at ordinary temperatures so that direct polarization can be made first and invert polarization later. The method, although sometimes useful, is far from ideal, as shown by Browne, Pellet, and others.

VI. Deerr Sulfuric Acid Inversion Modification.[17] This ingenious process has the advantage of defecating and inverting without leaving any added substances in the solutions used for either the direct or invert polarization; furthermore, the inversion is carried out on a boiling water bath for 20 minutes, which is more convenient and less subject to variation than the usual processes. It is also an excellent method of preparation of solutions for copper reduction.

PROCESS. *Defecation.* Defecate a normal quantity of the sample in a 100-ml. flask with 30 ml. of *0.4 N barium hydroxide solution* and 10 ml. of an acid mixture containing in 1 liter 222 g. of crystallized *aluminum sulfate* and 200 ml. of *1.0 N sulfuric acid*. The two solutions neutralize each other with the formation of aluminum hydroxide and barium sulfate.

Inversion. For inversion, heat 50 ml. of the defecated solution with 10 ml. of the *acid*

mixture on a boiling water bath at 97° for 20 minutes, then cool, add 30 ml. of *barium hydroxide solution*, make up to 100 ml., mix, filter, and polarize.

VII. Scheibler Double Dilution Modification.[18] *Double Polarization to Correct for Bulk of Precipitate*. When defecation is carried out by Horne's method, using anhydrous lead subacetate after making up to the mark, no correction for the bulk of the precipitate is necessary; nor is a correction necessary when lead subacetate solution is used and the bulk of the precipitate is small. When the bulk is large, as in blackstrap molasses, and correction is deemed necessary, Scheibler's modification is satisfactory for most purposes. It is briefly as follows: Weigh out normal portions of the sample into 100- and 200-ml. graduated flasks, add a slight excess of *lead subacetate*, make up to the mark, filter, polarize, and calculate the corrected polarization by multiplying the polarization of the dilute solution by 4 and subtracting the polarization of the more concentrated solution.

VIII. Pellet and Lemeland Alkaline-Peroxide Modification.[19] This method, depending on the destruction of invert sugar, is specially suited for cane molasses, but is applicable to other saccharine materials containing about the same amount of reducing sugars.

Process. Prepare a solution containing 5% or less of reducing sugars and pipet 50 ml. into a 300-ml. graduated flask. Add 7.5 ml. of *sodium hydroxide solution* (sp.gr. 1.325), 75 ml. of *hydrogen peroxide solution* (3% by weight), and 60 ml. of water. Shake, heat in a boiling water bath for 20 minutes, cool, neutralize with *acetic acid*, and clarify with a slight excess of *lead subacetate solution*. Make up to the mark, shake, and filter. Polarize in a 200- or 400-mm. tube, then place 50 ml. in a flask graduated at 50 and 55 ml., add 1 ml. of *glacial acetic acid*, make up to the 55-ml. mark, and polarize again. The second polarization corrected for dilution, if different from the first, should be used in the calculation.

Note. Fribourg [20] obtained by this modification results on cane molasses agreeing closely with those by the Clerget method (with neutralization), also the sulfur dioxide, the Stanek hydrochloric acid-urea, and the Ogilvie invertase modifications.

IX. Cross and Taggart Modification.[21] Place a normal weight of the sample in a 100-ml. graduated flask, make up to the mark, mix, and transfer 50 ml. to another 100-ml. flask. Add 6.3 ml. of *sodium hydroxide solution* (sp.gr. 1.325), 7.5 ml. of *hydrogen peroxide solution* (30% by weight), moderate the reaction by immersion in ice water, and after the evolution of the gas has almost ceased immerse for 20 minutes in a bath at 50°. Cool, acidify slightly with *acetic acid*, and make up to the mark. Clarify with *dry lead subacetate*, filter, and polarize in a 400-mm. tube, thus obtaining the percentage of sucrose without calculation.

X. Various Modifications. Numerous attempts have been made to overcome the inaccuracies of Clerget's method. Some of the modifications in removing certain sources of error have added new ones, others have employed reagents not readily procurable, and others still, that seem promising, have been given scanty attention by chemists content with standard methods. Although recognizing that petty changes in the manipulation have caused complications and a division of credit due Clerget, certain ingenious modifications are perhaps worthy of further study.

Kjeldahl Direct and Invertase Inversion Polariscopic Method.[22] Kjeldahl first proposed invertase as the hydrolyzing agent, thus avoiding the errors due to acid inversion. The method has been modified by several English and American chemists and adopted as an Official A.O.A.C. Method in the following form.

Reagent. *Invertase Solution*. Invertase suitable for the purpose is obtainable from

supply houses. It may also be prepared and purified in the laboratory by the collodion ultrafiltration method devised by Reynolds.[23] In either case, the purity of the preparation should be established by a determination on pure sucrose.

PROCESS. *Direct Polarization.* Dissolve 52 g. of the sample in a 200-ml. flask, clarify in the usual manner, avoiding an excess, dilute to volume, mix, and filter, rejecting the first portions of the filtrate. Delead with *anhydrous sodium carbonate*, added in small portions, avoiding an excess, filter, and reject the first portions that run through. Pipet 50 ml. into a 100-ml. flask, dilute to volume, and polarize in a 200-mm. tube.

Invert Polarization. Ascertain by titration the volume of *acetic acid* necessary to render 50 ml. of the deleaded solution distinctly acid to *methyl red* and add that volume to a fresh 50-ml. portion in a 100-ml. flask, also add 5 ml. of the *invertase solution*, fill nearly to the mark, and let stand overnight at 20° or a little above. Cool to about 20°, fill exactly to the mark, mix, and polarize at 20° in a 200-mm. tube. Check the completeness of the inversion by taking another reading several hours later.

If rapid inversion is desired, proceed as above, but add 10 ml. (instead of 5 ml.) of the *invertase solution* and heat at 55 to 60° for 15 minutes (instead of at 20° overnight) with shaking.

CALCULATION. Obtain the percentage of sucrose (S) by the following formula:

$$S = \frac{100P - P'}{142.1 + 0.073(g - 13) - 0.5t}$$

in which P and P' are the direct and invert polariscopic readings (normal solutions) respectively, g is the grams per 100 ml. of total solids (derived from the sample) of the invert solution, and t is the temperature.

General Notes on Preparation of Polarizing Solutions. *Deleading.* A moderate excess of lead subacetate is practically without influence on the polarization of sucrose; it is only when in large excess, as shown by Bates and Blake,[24] that it introduces a considerable error. If, however, the amount of reducing sugars is such as to warrant double polarization, the removal of the excess of lead is essential, since it forms with levulose a soluble compound (lead fructosate) which has a strong plus polarization and as a consequence the direct polarization, owing to reducing sugars, will be greater than that of the same sugars after the inversion process, during which the levulose is liberated by the acid from its lead combination.

Deleading is ordinarily accomplished by adding *powdered ammonium oxalate, sodium sulfate*, or *sodium carbonate* in small successive amounts with shaking, until no further precipitation takes place, and filtering. By proceeding in this manner, the volume is not affected, but the solution may be left alkaline, in which case neutralizing with *acetic acid* and making up to a new volume is recommended.

Pellet deleads with a strong *sulfurous acid solution* which serves also to neutralize the alkalinity and decolorize.

Ogilvie[25] passes *sulfur dioxide gas* into 50 ml. of the solution for 10 minutes or makes 50 ml. up to 100 ml. with a *saturated sulfurous acid solution*. Using these amounts, which are more than Pellet recommends, he finds that the direct polarization of the filtered solution is usually comparable with the invert polarization following the Clerget-Herzfeld method without the sulfurous acid treatment, although filtering from the lead chloride which separates. He notes that when invert sugar is present the levorotation of the levulose is increased about 15% in the presence of sulfurous acid, whereas in the presence of hydrochloric acid it is little changed.

Bone Black (animal charcoal) or blood charcoal is an efficient decolorizer in analytical processes, as in sugar refining, but absorbs sucrose to such a degree as to render it entirely unfit for accurate work. In certain cases, where sufficient decolorization cannot be effected by other means, it is employed with the understanding that the results obtained are only approximate, unless the coefficient of absorp-

tion is determined for the particular lot of bone black under the conditions used. The error due to sucrose absorption may be reduced by pouring the solution of the sample through finely divided bone black on a filter and polarizing the successive portions that run through until the increase between different portions is inconsiderable.

Shilstone and Hartogh [26] shake 50 g. of bone black with 200 ml. of 5% sucrose solution, allow to settle, wash by decantation until the washings polarize zero in a 200-mm. tube, and dry the bone black on the steam bath. The material thus saturated with sucrose, when used as a clarifier for molasses, is said not to influence the polarization regardless of the amount employed.

Suitable animal or blood charcoal, treated with acid and washed, may be obtained from the dealer or prepared as follows.[27] Digest the finely ground commercial product for several hours with dilute hydrochloric acid at room temperature, filter, wash, dry, and heat to dull redness in a covered crucible. Bottle while warm.

Neutralization after Inversion is desirable in the presence of considerable amounts of reducing sugars since *d*-fructose gives a much stronger minus polarization in the solution strongly acid with hydrochloric acid than in the direct polarization where the free acid present is a trace of acetic. Furthermore, the presence of the free mineral acid changes the polarization of amino acids, often present in molasses and juices, from minus to plus or increases the plus polarization. When neutralization is carried out in conjunction with the Clerget-Herzfeld process, the factor 141.7 should be used instead of 142.66.

The chief objection to this method is that whereas one factor (free hydrochloric acid) in the invert polarization differing from that in the direct is removed, another (sodium chloride) is introduced and, although the amended formula takes this into consideration as regards the invert sugar formed from sucrose, it does not correct for the difference in the polarization of the reducing sugars before inversion, where sodium chloride is absent, from that after inversion, where it is present.

Invert Sugar

Herzfeld Copper Reduction Gravimetric Method for High Grade Raw and Brown Sugar.[28] The invert sugar content should not exceed 1.5%, but the sucrose may reach 99.

Reagents. *Fehling-Soxhlet Solutions 1 and 2* (Part I, C6*a*, Reducing Sugars).

Process. *Clarification.* Weigh 44 g. of the sugar into a 200-ml. volumetric flask, dissolve in about 100 ml. of water, add *normal lead acetate solution sufficient* to remove impurities, fill to the mark, shake, allow to settle, and filter.

Deleading. Remove 100 ml. of the filtrate to a 110-ml. volumetric flask, precipitate the excess of lead with *sodium carbonate* or *sodium sulfate* (or else a solution of sodium phosphate or potassium oxalate), make up to the 110-ml. mark, shake, allow to settle, and filter.

If preferred, use 40 g. of the sample, delead with dry *sodium* or *potassium oxalate* in the 200-ml. flask, and proceed with the copper reduction after making up to 200 ml. and filtering.

Copper Reduction. Heat to boiling a mixture of 25 ml. each of *Fehling-Soxhlet solutions 1 and 2*, add 50 ml. of the clarified filtrate (equivalent to 10 g. of the sample), bring to a boil, and boil for 2 minutes. Add 100 ml. of cold, recently boiled water, filter at once, and proceed as in the Munson and Walker Method (Part I, C6*a*). Weigh as metallic copper or cuprous oxide.

Calculation. From the Herzfeld table obtain directly the per cent of invert sugar.

Meissl and Hiller Copper Reduction Gravimetric Method.[29] The method is applicable only to products containing at least 1% of invert sugar.

Process. *Clarification.* Dissolve 44 g. of the sugar in water, clarify, and delead as described for the Herzfeld method.

INVERT SUGAR FROM COPPER (HERZFELD)

Copper (Cu)	Invert Sugar	Copper (Cu)	Invert Sugar	Copper (Cu)	Invert Sugar	Copper (Cu)	Invert Sugar	Copper (Cu)	Invert Sugar	Copper (Cu)	Invert Sugar
mg.	%	mg.	%	mg.	%	mg.	%	mg.	%	mg.	%
50	0.050	95	0.271	140	0.503	185	0.764	230	1.013	275	1.271
51	.054	96	.277	141	.509	186	.770	231	.018	276	.276
52	.058	97	.283	142	.515	187	.775	232	.024	277	.282
53	.062	98	.288	143	.521	188	.781	233	.030	278	.288
54	.066	99	.294	144	.527	189	.786	234	.036	279	.294
55	.070	100	.300	145	.533	190	.792	235	.041	280	.299
56	.074	101	.305	146	.538	191	.797	236	.047	281	.305
57	.078	102	.310	147	.544	192	.803	237	.053	282	.311
58	.082	103	.315	148	.550	193	.808	238	.058	283	.317
59	.086	104	.320	149	.556	194	.814	239	.064	284	.322
60	.090	105	.325	150	.562	195	.819	240	.070	285	.328
61	.094	106	.330	151	.568	196	.825	241	.076	286	.334
62	.098	107	.335	152	.574	197	.830	242	.081	287	.339
63	.103	108	.340	153	.580	198	.836	243	.087	288	.345
64	.108	109	.346	154	.586	199	.841	244	.093	289	.351
65	.113	110	.351	155	.592	200	.847	245	.099	290	.357
66	.118	111	.356	156	.598	201	.852	246	.104	291	.362
67	.123	112	.361	157	.604	202	.858	247	.110	292	.368
68	.128	113	.366	158	.609	203	.863	248	.116	293	.374
69	.133	114	.371	159	.615	204	.869	249	.122	294	.380
70	.138	115	.376	160	.621	205	.874	250	.127	295	.385
71	.143	116	.381	161	.627	206	.880	251	.133	296	.391
72	.148	117	.386	162	.633	207	.885	252	.139	297	.397
73	.152	118	.392	163	.639	208	.891	253	.144	298	.403
74	.157	119	.397	164	.645	209	.896	254	.150	299	.408
75	.162	120	.402	165	.651	210	.902	255	.156	300	.414
76	.167	121	.407	166	.657	211	.907	256	.162	301	.420
77	.172	122	.412	167	.663	212	.913	257	.167	302	.425
78	.177	123	.417	168	.669	213	.918	258	.173	303	.431
79	.182	124	.423	169	.675	214	.924	259	.179	304	.437
80	.187	125	.428	170	.680	215	.929	260	.185	305	.443
81	.192	126	.433	171	.686	216	.935	261	.190	306	.448
82	.197	127	.438	172	.692	217	.940	262	.196	307	.454
83	.202	128	.443	173	.698	218	.946	263	.202	308	.460
84	.208	129	.448	174	.704	219	.951	264	.207	309	.466
85	.213	130	.453	175	.709	220	.957	265	.213	310	.471
86	.219	131	.458	176	.715	221	.962	266	.219	311	.477
87	.225	132	.463	177	.720	222	.968	267	.225	312	.483
88	.231	133	.468	178	.726	223	.973	268	.231	313	.489
89	.236	134	.473	179	.731	224	.979	269	.236	314	.494
90	.242	135	.478	180	.737	225	.984	270	.242	315	.500
91	.248	136	.483	181	.742	226	.990	271	.248		
92	.254	137	.488	182	.748	227	.996	272	.253		
93	.260	138	.493	183	.753	228	1.001	273	.259		
94	.265	139	.498	184	.759	229	.007	274	.265		

Preliminary Copper Reduction. Pipet, into each of 5 large test tubes, volumes of the clarified and deleaded sugar solution ranging from 1 to 5 ml. To each, add 5 ml. of a mixture of equal parts of *Fehling-Soxhlet solutions 1 and 2*, heat to boiling, boil 2 minutes, and filter. Compare the intensity of the blue color in the filtrates.

Final Copper Reduction. Multiply by 20 the number of milliliters of sugar solution corresponding to the filtrate of the lightest blue color and pipet that number of milliliters of the clarified and deleaded solution into a 100-ml. volumetric flask, dilute to the mark, shake, and proceed with 50 ml. as in the Herzfeld method.

Calculation. The percentage of invert sugar is obtained by the formula CuF/W in which Cu is the weight of copper found, W is the weight of sample in the 50 ml. used for the copper reduction, and F is the factor derived from the accompanying table. In the table, A is the approximate weight of invert sugar obtained by dividing the weight of copper found by 2; S is the approximate per cent of sucrose in the mixed sugars, obtained by solving the equation $S = 100P/(P + y)$ in which P is the polarization and y is the approximate per cent of invert sugar, that is, $A \times (100/W)$; and I is the approximate per cent of invert sugar in the mixed sugars, obtained by solving the equation $I = 100 - S$.

It should be noted that both y and I represent the approximate per cent of invert sugar and that in solving the equations the values for the two are slightly different. Only the value for I should be applied in the table, that for y serving merely in obtaining an approximate value for S.

Factors for the Calculation of Invert (Meissl and Hiller)

(For use in Conjunction with the Formula CuF/W)

Ratio of Sucrose to Invert Sugar ($S : I$)	Factors (F) for Approximate Weights of Invert Sugar (A)						
	200 mg.	175 mg.	150 mg.	125 mg.	100 mg.	75 mg.	50 mg.
0 : 100	56.4	55.4	54.5	53.8	53.2	53.0	53.0
10 : 90	56.3	55.3	54.4	53.8	53.2	52.9	52.9
20 : 80	56.2	55.2	54.3	53.7	53.2	52.7	52.7
30 : 70	56.1	55.1	54.2	53.7	53.2	52.6	52.6
40 : 60	55.9	55.0	54.1	53.6	53.1	52.5	52.4
50 : 50	55.7	54.9	54.0	53.5	53.1	52.3	52.2
60 : 40	55.6	54.7	53.8	53.2	52.8	52.1	51.9
70 : 30	55.5	54.5	53.5	52.9	52.5	51.9	51.6
80 : 20	55.4	54.3	53.3	52.7	52.2	51.7	51.3
90 : 10	54.6	53.6	53.1	52.6	52.1	51.6	51.2
91 : 9	54.1	53.6	52.6	52.1	51.6	51.2	50.7
92 : 8	53.6	53.1	52.1	51.6	51.2	50.7	50.3
93 : 7	53.6	53.1	52.1	51.2	50.7	50.3	49.8
94 : 6	53.1	52.6	51.6	50.7	50.3	49.8	48.9
95 : 5	52.6	52.1	51.2	50.3	49.4	48.9	48.5
96 : 4	52.1	51.2	50.7	49.8	48.9	47.7	46.9
97 : 3	50.7	50.3	49.8	48.9	47.7	46.2	45.1
98 : 2	49.9	48.9	48.5	47.3	45.8	43.3	40.0
99 : 1	47.7	47.3	46.5	45.1	43.3	41.2	38.1

Sucrose; Dextrose; Levulose; Maltose

See also Sugar in Part I, C6*a*, where chemical methods, both unified and individual, for the determination of sucrose, dextrose, levulose, and maltose, together with additional methods for the determination of invert sugar, are described. Lactose is determined by methods given in Part II, G1.

Ash

Incineration Gravimetric Method. Ignite below redness in a muffle furnace 5 g. of the sample, or the residue from the direct determination of moisture, in a platinum, quartz, or porcelain dish of good quality. Cool in a desiccator and weigh. Add *nitric acid* if necessary to secure a white ash. See also methods in Part I, C2*f*, and Part II, A2.

Lead

Fischer Dithizone Colorimetric Method. See Part I, C8*b*.

NOTE. Gray [30] gives detailed instructions for the preparation of lead-free reagents, the routine determination of lead in sugar and sugar products, and illustrations showing vacuum pipet, batteries of separatory funnels and Nessler tubes, and other apparatus.

3. MOLASSES AND SIRUP

The term *molasses* is applied to the concentrated liquid separated in the sugar house from the massecuite in the centrifuge, also to the final liquid from which no more sugar can be separated. It is this latter product that enters into commerce in different grades.

According to the U. S. Standards, light and dark molasses must contain 62 and 55% respectively of sucrose and not more than 25% of moisture.

Beet molasses is not edible, even for farm animals; it is used for the manufacture of alcohol.

The word *sirup* has two meanings in the sugar industry. *Cane sirup*, obtained by the evaporation of cane juice to a consistency short of crystallization, is a product of superior excellence comparable with maple sirup. *Refinery sirup* is the by-product of sugar refineries; it contains the reducing sugars, coloring matter, and other constituents removed from raw sugar in the manufacture of white sugar. It contains much less sucrose than the concentrated cane juice.

Massecuite is analyzed by the same methods as molasses and sirup, but such analyses are of interest only to the sugar producer.

SOLIDS

(Water)

Custom dictates that the results of drying solid materials be calculated as moisture and of liquids or semi-liquids as solids. By using an absorbent, such as granular pumice stone, asbestos, or sand, the drying is facilitated. The decomposition of the levulose of invert sugar at 100° is prevented by drying at 70°.

Direct Gravimetric Method. The air oven method described under E2 above may be used where extreme accuracy is not essential.

A.O.A.C. Official Sand Gravimetric Method.[31] Weigh a charge equal to about 1 g. of dry matter into a round dish 55 mm. in diameter and 40 mm. deep, containing 25 to 30 g. of 40 mesh *acid-treated sand* and a short stirring rod. Dry over a steam bath with stirring for 15 to 20 minutes, then in a vacuum oven at 70° under a pressure not exceeding 50 mm. of mercury for 18 hours, cool, and weigh. Repeat the drying until the loss in 2 hours does not exceed 2 mg.

In the absence of levulose or other readily decomposable substances, dry for 8 to 10 hours at atmospheric pressure in an ordinary water oven, repeating the drying until the loss in 1 hour does not exceed 2 mg.

Browne Vacuum Oven Gravimetric Method.[32] Weigh a perforated cylinder, such as is used in milk analysis, containing ignited woolly asbestos so packed against the sides as to leave a central cavity in the upper portion. Introduce into the central cavity, from a weighing bottle fitted with a medicine dropper, 5 ml. of the solution of the sample containing not more than 25% of solids. Dry in a vacuum oven heated at 70°, at first in a current of air to remove the excess of moisture, then in a vacuum of 25 mm. After 8 hours, cool and weigh the cylinder, then heat for 2-hour periods until the weight is practically constant.

If no appreciable amount of levulose is present, the drying may be carried out in a current of air at the temperature of boiling water.

Perforated cylinders, made of platinum, are ideal for determining moisture and ash on the same weighed portion.

Density Method. Determine the density at 20° 20° by a Brix saccharimeter (a hydrometer with a specific gravity scale) or a

Sucrose Per Cent by Weight from Apparent Specific Gravity at $\frac{20°}{20°}$ C. (Domke) *

Degrees Brix or Sucrose Per Cent	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
0	1.0000	1.0004	1.0008	1.0012	1.0016	1.0019	1.0023	1.0027	1.0031	1.0035
1	.0039	.0043	.0047	.0051	.0055	.0058	.0062	.0066	.0070	.0074
2	.0078	.0082	.0086	.0090	.0094	.0098	.0102	.0106	.0109	.0113
3	.0117	.0121	.0125	.0129	.0133	.0137	.0141	.0145	.0149	.0153
4	.0157	.0161	.0165	.0169	.0173	.0177	.0181	.0185	.0189	.0193
5	.0197	.0201	.0205	.0209	.0213	.0217	.0221	.0225	.0229	.0233
6	.0237	.0241	.0245	.0249	.0253	.0257	.0261	.0265	.0269	.0273
7	.0277	.0281	.0285	.0289	.0294	.0298	.0302	.0306	.0310	.0314
8	.0318	.0322	.0326	.0330	.0334	.0338	.0343	.0347	.0351	.0355
9	.0359	.0363	.0367	.0371	.0375	.0380	.0384	.0388	.0392	.0396
10	.0400	.0404	.0409	.0413	.0417	.0421	.0425	.0429	.0433	.0438
11	.0442	.0446	.0450	.0454	.0459	.0463	.0467	.0471	.0475	.0480
12	.0484	.0488	.0492	.0496	.0501	.0505	.0509	.0513	.0517	.0522
13	.0526	.0530	.0534	.0539	.0543	.0547	.0551	.0556	.0560	.0564
14	.0568	.0573	.0577	.0581	.0585	.0589	.0594	.0598	.0603	.0607
15	.0611	.0615	.0620	.0624	.0628	.0633	.0637	.0641	.0646	.0650
16	.0654	.0659	.0663	.0667	.0672	.0676	.0680	.0685	.0689	.0693
17	.0698	.0702	.0706	.0711	.0715	.0719	.0724	.0728	.0733	.0737
18	.0741	.0746	.0750	.0755	.0759	.0763	.0768	.0772	.0777	.0781
19	.0785	.0790	.0794	.0799	.0803	.0807	.0812	.0816	.0821	.0825
20	.0830	.0834	.0839	.0843	.0848	.0852	.0856	.0861	.0865	.0870
21	.0874	.0879	.0883	.0888	.0892	.0897	.0901	.0905	.0910	.0915
22	.0919	.0924	.0928	.0933	.0937	.0942	.0946	.0951	.0956	.0960
23	.0965	.0969	.0974	.0978	.0983	.0987	.0992	.0997	.1001	.1006
24	.1010	.1015	.1020	.1024	.1029	.1033	.1038	.1043	.1047	.1052
25	.1056	.1061	.1066	.1070	.1075	.1079	.1084	.1089	.1093	.1098
26	.1103	.1107	.1112	.1117	.1121	.1126	.1131	.1135	.1140	.1145
27	.1149	.1154	.1159	.1163	.1168	.1173	.1178	.1182	.1187	.1192
28	.1196	.1201	.1206	.1210	.1215	.1220	.1225	.1229	.1234	.1239
29	.1244	.1248	.1253	.1258	.1263	.1267	.1272	.1277	.1282	.1287
30	.1291	.1296	.1301	.1306	.1311	.1315	.1320	.1325	.1330	.1334
31	.1339	.1344	.1349	.1354	.1359	.1363	.1368	.1373	.1378	.1383
32	.1388	.1393	.1397	.1402	.1407	.1412	.1417	.1422	.1427	.1432
33	.1436	.1441	.1446	.1451	.1456	.1461	.1466	.1471	.1476	.1481
34	.1486	.1490	.1495	.1500	.1505	.1510	.1515	.1520	.1525	.1530
35	.1535	.1540	.1545	.1550	.1555	.1560	.1565	.1570	.1575	.1580
36	.1585	.1590	.1595	.1600	.1605	.1610	.1615	.1620	.1625	.1630
37	.1635	.1640	.1645	.1650	.1655	.1660	.1665	.1670	.1675	.1680
38	.1685	.1690	.1696	.1701	.1706	.1711	.1716	.1721	.1726	.1731
39	.1736	.1741	.1746	.1752	.1757	.1762	.1767	.1772	.1777	.1782
40	.1787	.1793	.1798	.1803	.1808	.1813	.1818	.1824	.1829	.1834
41	.1839	.1844	.1849	.1855	.1860	.1865	.1870	.1875	.1881	.1886
42	.1891	.1896	.1901	.1907	.1912	.1917	.1922	.1928	.1933	.1938
43	.1943	.1949	.1954	.1959	.1964	.1970	.1975	.1980	.1985	.1991
44	.1996	.2001	.2007	.2012	.2017	.2023	.2028	.2033	.2039	.2044

* *Z. Ver. deut. Zucker-Ind.* 1912, **62**, 306, via *Methods of Analysis, A.O.A.C.* Calculated from Kaiserliche-Normal-Eichungs-Kommission table and accepted by the International Commission for Unifying Methods of Sugar Analysis.

Sucrose Per Cent by Weight from Apparent Specific Gravity at $\frac{20^\circ}{20^\circ}$ C.

Concluded

Degrees Brix or Sucrose Per Cent	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
45	1.2049	1.2054	1.2060	1.2065	1.2070	1.2076	1.2081	1.2087	1.2092	1.2097
46	.2102	.2108	.2113	.2118	.2124	.2129	.2135	.2140	.2146	.2151
47	.2156	.2162	.2167	.2173	.2178	.2184	.2189	.2194	.2200	.2205
48	.2211	.2216	.2222	.2227	.2232	.2238	.2243	.2249	.2254	.2260
49	.2265	.2271	.2276	.2282	.2287	.2293	.2298	.2304	.2309	.2315
50	.2320	.2326	.2331	.2337	.2342	.2348	.2353	.2359	.2364	.2370
51	.2376	.2381	.2387	.2392	.2398	.2403	.2409	.2415	.2420	.2426
52	.2431	.2437	.2442	.2448	.2454	.2459	.2465	.2471	.2476	.2482
53	.2487	.2493	.2499	.2504	.2510	.2516	.2521	.2527	.2533	.2538
54	.2544	.2550	.2555	.2561	.2567	.2572	.2578	.2584	.2589	.2595
55	.2601	.2606	.2612	.2618	.2624	.2629	.2635	.2641	.2647	.2652
56	.2658	.2664	.2670	.2675	.2681	.2687	.2693	.2698	.2704	.2710
57	.2716	.2721	.2727	.2733	.2739	.2745	.2750	.2756	.2762	.2768
58	.2774	.2779	.2785	.2791	.2797	.2803	.2809	.2815	.2821	.2826
59	.2832	.2838	.2844	.2850	.2856	.2861	.2867	.2873	.2879	.2885
60	.2891	.2897	.2903	.2909	.2914	.2920	.2926	.2932	.2938	.2944
61	.2950	.2956	.2962	.2968	.2974	.2980	.2986	.2992	.2998	.3004
62	.3010	.3015	.3021	.3027	.3033	.3039	.3045	.3051	.3057	.3063
63	.3069	.3075	.3081	.3087	.3093	.3100	.3106	.3112	.3118	.3124
64	.3130	.3136	.3142	.3148	.3154	.3160	.3166	.3172	.3178	.3184
65	.3190	.3197	.3203	.3209	.3215	.3221	.3227	.3233	.3239	.3245
66	.3252	.3258	.3264	.3270	.3276	.3282	.3288	.3295	.3301	.3307
67	.3313	.3319	.3325	.3332	.3338	.3344	.3350	.3356	.3363	.3369
68	.3375	.3381	.3387	.3394	.3400	.3406	.3412	.3418	.3425	.3431
69	.3437	.3443	.3450	.3456	.3462	.3468	.3475	.3481	.3487	.3494
70	.3500	.3506	.3512	.3519	.3525	.3531	.3538	.3544	.3550	.3557
71	.3563	.3569	.3575	.3582	.3588	.3594	.3601	.3607	.3614	.3620
72	.3626	.3633	.3639	.3645	.3652	.3658	.3664	.3671	.3677	.3684
73	.3690	.3696	.3703	.3709	.3716	.3722	.3729	.3735	.3741	.3748
74	.3754	.3761	.3767	.3774	.3780	.3786	.3793	.3799	.3806	.3812
75	.3819	.3825	.3832	.3838	.3845	.3851	.3858	.3864	.3871	.3877
76	.3884	.3890	.3897	.3903	.3910	.3916	.3923	.3929	.3936	.3942
77	.3949	.3955	.3962	.3969	.3975	3982	.3988	.3995	.4001	.4008
78	.4015	.4021	.4028	.4034	.4041	.4048	.4054	.4061	.4067	.4074
79	.4081	.4087	.4094	.4101	.4107	.4114	.4121	.4127	.4134	.4140
80	.4147	.4154	.4160	.4167	.4174	.4180	.4187	.4194	4201	.4207
81	.4214	.4221	.4227	.4234	.4241	.4247	.4254	.4261	.4268	.4274
82	.4281	.4288	.4295	.4301	.4308	.4315	.4322	.4328	.4335	.4342
83	.4349	.4355	.4362	.4369	.4376	.4383	.4389	.4396	.4403	.4410
84	.4417	.4423	.4430	.4437	.4444	.4451	.4458	.4464	.4471	.4478
85	.4485	.4492	.4499	.4505	.4512	.4519	.4526	.4533	.4540	.4547
86	.4554	.4560	.4567	.4574	.4581	.4588	.4595	.4602	.4609	.4616
87	.4623	.4629	.4636	.4643	.4650	.4657	.4664	.4671	.4678	.4685
88	.4692	.4699	.4706	.4713	.4720	.4727	.4734	.4741	.4748	.4755
89	.4762	.4769	.4776	.4783	.4790	.4797	.4804	.4811	.4818	.4825
90	.4832									

Corrections of Saccharimeter Readings to 20° C. (N.B.S.) *

Temperature ° C.	Observed Per Cent of Sugar													
	0	5	10	15	20	25	30	35	40	45	50	55	60	70
	Subtract from Observed Per Cent													
0	0.30	0.49	0.65	0.77	0.89	0.99	1.08	1.16	1.24	1.31	1.37	1.41	1.44	1.49
5	0.36	0.47	0.56	0.65	0.73	0.80	0.86	0.91	0.97	1.01	1.05	1.08	1.10	1.14
10	0.32	0.38	0.43	0.48	0.52	0.57	0.60	0.64	0.67	0.70	0.72	0.74	0.75	0.77
11	0.31	0.35	0.40	0.44	0.48	0.51	0.55	0.58	0.60	0.63	0.65	0.66	0.68	0.70
12	0.29	0.32	0.36	0.40	0.43	0.46	0.50	0.52	0.54	0.56	0.58	0.59	0.60	0.62
13	0.26	0.29	0.32	0.35	0.38	0.41	0.44	0.46	0.48	0.49	0.51	0.52	0.53	0.55
14	0.24	0.26	0.29	0.31	0.34	0.36	0.38	0.40	0.41	0.42	0.44	0.45	0.46	0.47
15	0.20	0.22	0.24	0.26	0.28	0.30	0.32	0.33	0.34	0.36	0.36	0.37	0.38	0.39
16	0.17	0.18	0.20	0.22	0.23	0.25	0.26	0.27	0.28	0.28	0.29	0.30	0.31	0.32
17	0.13	0.14	0.15	0.16	0.18	0.19	0.20	0.20	0.21	0.21	0.22	0.23	0.23	0.24
18	0.09	0.10	0.10	0.11	0.12	0.13	0.13	0.14	0.14	0.14	0.15	0.15	0.15	0.16
19	0.05	0.05	0.05	0.06	0.06	0.06	0.07	0.07	0.07	0.07	0.08	0.08	0.08	0.08
17.5	0.11	0.12	0.12	0.14	0.15	0.16	0.16	0.17	0.17	0.18	0.18	0.19	0.19	0.20
15.56	0.18	0.20	0.22	0.24	0.26	0.28	0.29	0.30	0.30	0.32	0.33	0.33	0.34	0.34
	Add to Observed Per Cent													
21	0.04	0.05	0.06	0.06	0.06	0.07	0.07	0.07	0.07	0.08	0.08	0.08	0.08	0.09
22	0.10	0.10	0.11	0.12	0.12	0.13	0.14	0.14	0.15	0.15	0.16	0.16	0.16	0.16
23	0.16	0.16	0.17	0.17	0.19	0.20	0.21	0.21	0.22	0.23	0.24	0.24	0.24	0.24
24	0.21	0.22	0.23	0.24	0.26	0.27	0.28	0.29	0.30	0.31	0.32	0.32	0.32	0.32
25	0.27	0.28	0.30	0.31	0.32	0.34	0.35	0.36	0.38	0.38	0.39	0.39	0.40	0.39
26	0.33	0.34	0.36	0.37	0.40	0.40	0.42	0.44	0.46	0.47	0.47	0.48	0.48	0.48
27	0.40	0.41	0.42	0.44	0.46	0.48	0.50	0.52	0.54	0.54	0.55	0.56	0.56	0.56
28	0.46	0.47	0.49	0.51	0.54	0.56	0.58	0.60	0.61	0.62	0.63	0.64	0.64	0.64
29	0.54	0.55	0.56	0.59	0.61	0.63	0.66	0.68	0.70	0.70	0.71	0.72	0.72	0.72
30	0.61	0.62	0.63	0.66	0.68	0.71	0.73	0.76	0.78	0.78	0.79	0.80	0.80	0.81
35	0.99	1.01	1.02	1.06	1.10	1.13	1.16	1.18	1.20	1.21	1.22	1.22	1.23	1.22
40	1.42	1.45	1.47	1.51	1.54	1.57	1.60	1.62	1.64	1.65	1.65	1.65	1.66	1.65
45	1.91	1.94	1.96	2.00	2.03	2.05	2.07	2.09	2.10	2.10	2.10	2.10	2.10	2.08
50	2.46	2.48	2.50	2.53	2.56	2.57	2.58	2.59	2.59	2.58	2.58	2.57	2.56	2.52
55	3.05	3.07	3.09	3.12	3.12	3.12	3.12	3.11	3.10	3.08	3.07	3.05	3.03	2.97
60	3.69	3.72	3.73	3.73	3.72	3.70	3.67	3.65	3.62	3.60	3.57	3.54	3.50	3.43
65	4.4	4.4	4.4	4.4	4.4	4.4	4.3	4.2	4.2	4.1	4.1	4.0	4.0	3.9
70	5.1	5.1	5.1	5.0	5.0	5.0	4.9	4.8	4.8	4.7	4.7	4.6	4.6	4.4
75	6.1	6.0	6.0	5.9	5.8	5.8	5.7	5.6	5.5	5.4	5.4	5.3	5.2	5.0
80	7.1	7.0	7.0	6.9	6.8	6.7	6.6	6.4	6.3	6.2	6.1	6.0	5.9	5.6

* *U. S. Dept. Com. and Lab., Natl. Bur. Standards* 1913, *Circ.* **44**, 129. Calculated from data of Plato (*Wiss. Abh. der Kaiserlichen Normal-Eichungs-Kommission* 1900, **2**, 140), assuming the instrument to be of Jena 16 [III] glass. The table yields only approximate results when the temperature differs much from the standard temperature or from the temperature of the surrounding air.

Sucrose Per Cent by Weight from Refractive Index at 20° C. (International Commission-A.O.A.C.) *

Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent
1.33299	0.0	1.34629	9.0	1.36053	18.0	1.3758	27.0	1.3920	36.0
.33328	0.2	.34660	9.2	.36086	18.2	.3761	27.2	.3924	36.2
.33357	0.4	.34691	9.4	.36119	18.4	.3765	27.4	.3928	36.4
.33385	0.6	.34721	9.6	.36152	18.6	.3768	27.6	.3931	36.6
.33414	0.8	.34752	9.8	.36185	18.8	.3772	27.8	.3935	36.8
.33443	1.0	.34783	10.0	.36218	19.0	.3775	28.0	.3939	37.0
.33472	1.2	.34814	10.2	.36251	19.2	.3779	28.2	.3943	37.2
.33501	1.4	.34845	10.4	.36284	19.4	.3782	28.4	.3947	37.4
.33530	1.6	.34875	10.6	.36318	19.6	.3786	28.6	.3950	37.6
.33559	1.8	.34906	10.8	.36351	19.8	.3789	28.8	.3954	37.8
.33588	2.0	.34937	11.0	.36384	20.0	.3793	29.0	.3958	38.0
.33617	2.2	.34968	11.2	.36417	20.2	.3797	29.2	.3962	38.2
.33646	2.4	.34999	11.4	.36451	20.4	.3800	29.4	.3966	38.4
.33675	2.6	.35031	11.6	.36484	20.6	.3804	29.6	.3970	38.6
.33704	2.8	.35062	11.8	.36518	20.8	.3807	29.8	.3974	38.8
.33733	3.0	.35093	12.0	.36551	21.0	.3811	30.0	.3978	39.0
.33762	3.2	.35124	12.2	.36585	21.2	.3815	30.2	.3982	39.2
.33792	3.4	.35156	12.4	.36618	21.4	.3818	30.4	.3986	39.4
.33821	3.6	.35187	12.6	.36652	21.6	.3822	30.6	.3989	39.6
.33851	3.8	.35219	12.8	.36685	21.8	.3825	30.8	.3993	39.8
.33880	4.0	.35250	13.0	.36719	22.0	.3829	31.0	.3997	40.0
.33909	4.2	.35282	13.2	.36753	22.2	.3833	31.2	.4001	40.2
.33939	4.4	.35313	13.4	.36787	22.4	.3836	31.4	.4005	40.4
.33968	4.6	.35345	13.6	.36820	22.6	.3840	31.6	.4008	40.6
.33998	4.8	.35376	13.8	.36854	22.8	.3843	31.8	.4012	40.8
.34027	5.0	.35408	14.0	.36888	23.0	.3847	32.0	.4016	41.0
.34057	5.2	.35440	14.2	.36922	23.2	.3851	32.2	.4020	41.2
.34087	5.4	.35472	14.4	.36956	23.4	.3854	32.4	.4024	41.4
.34116	5.6	.35503	14.6	.36991	23.6	.3858	32.6	.4028	41.6
.34146	5.8	.35535	14.8	.37025	23.8	.3861	32.8	.4032	41.8
.34176	6.0	.35567	15.0	.37059	24.0	.3865	33.0	.4036	42.0
.34206	6.2	.35599	15.2	.3709	24.2	.3869	33.2	.4040	42.2
.34236	6.4	.35631	15.4	.3713	24.4	.3872	33.4	.4044	42.4
.34266	6.6	.35664	15.6	.3716	24.6	.3876	33.6	.4048	42.6
.34296	6.8	.35696	15.8	.3720	24.8	.3879	33.8	.4052	42.8
.34326	7.0	.35728	16.0	.3723	25.0	.3883	34.0	.4056	43.0
.34356	7.2	.35760	16.2	.3726	25.2	.3887	34.2	.4060	43.2
.34386	7.4	.35793	16.4	.3730	25.4	.3891	34.4	.4064	43.4
.34417	7.6	.35825	16.6	.3733	25.6	.3894	34.6	.4068	43.6
.34447	7.8	.35858	16.8	.3737	25.8	.3898	34.8	.4072	43.8
.34477	8.0	.35890	17.0	.3740	26.0	.3902	35.0	.4076	44.0
.34507	8.2	.35923	17.2	.3744	26.2	.3906	35.2	.4080	44.2
.34538	8.4	.35955	17.4	.3747	26.4	.3909	35.4	.4084	44.4
.34568	8.6	.35988	17.6	.3751	26.6	.3913	35.6	.4088	44.6
.34599	8.8	.36020	17.8	.3754	26.8	.3916	35.8	.4092	44.8

* The values for whole per cents are those of the International Scale (*Intern. Sugar J.* 1937, **39**, 228); the fractional values were obtained by interpolation (*Methods of Analysis, A.O.A.C.*). Indices from 0 to 24% are given to five places of decimals for use with refractometers reading to the fifth place.

SUCROSE PER CENT BY WEIGHT FROM REFRACTIVE INDEX AT 20° C. (INTERNATIONAL COMMISSION-A.O.A.C.)—*Concluded*

Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent	Ref. Ind. 20° C.	Sucrose Per Cent
1.4096	45.0	1.4264	53.0	1.4441	61.0	1.4627	69.0	1.4825	77.0
.4100	45.2	.4268	53.2	.4446	61.2	.4631	69.2	.4830	77.2
.4104	45.4	.4272	53.4	.4450	61.4	.4636	69.4	.4835	77.4
.4109	45.6	.4277	53.6	.4455	61.6	.4641	69.6	.4840	77.6
.4113	45.8	.4281	53.8	.4459	61.8	.4646	69.8	.4845	77.8
.4117	46.0	.4285	54.0	.4464	62.0	.4651	70.0	.4850	78.0
.4121	46.2	.4289	54.2	.4468	62.2	.4656	70.2	.4855	78.2
.4125	46.4	.4294	54.4	.4473	62.4	.4661	70.4	.4860	78.4
.4129	46.6	.4298	54.6	.4477	62.6	.4666	70.6	.4865	78.6
.4133	46.8	.4303	54.8	.4482	62.8	.4671	70.8	.4871	78.8
.4137	47.0	.4307	55.0	.4486	63.0	.4676	71.0	.4876	79.0
.4141	47.2	.4311	55.2	.4491	63.2	.4681	71.2	.4881	79.2
.4145	47.4	.4316	55.4	.4495	63.4	.4685	71.4	.4886	79.4
.4150	47.6	.4320	55.6	.4500	63.6	.4690	71.6	.4891	79.6
.4154	47.8	.4325	55.8	.4504	63.8	.4695	71.8	.4896	79.8
.4158	48.0	.4329	56.0	.4509	64.0	.4700	72.0	.4901	80.0
.4162	48.2	.4333	56.2	.4514	64.2	.4705	72.2	.4906	80.2
.4166	48.4	.4338	56.4	.4518	64.4	.4710	72.4	.4912	80.4
.4171	48.6	.4342	56.6	.4523	64.6	.4715	72.6	.4917	80.6
.4175	48.8	.4347	56.8	.4527	64.8	.4720	72.8	.4922	80.8
.4179	49.0	.4351	57.0	.4532	65.0	.4725	73.0	.4927	81.0
.4183	49.2	.4355	57.2	.4537	65.2	.4730	73.2	.4933	81.2
.4187	49.4	.4360	57.4	.4541	65.4	.4735	73.4	.4938	81.4
.4192	49.6	.4364	57.6	.4546	65.6	.4740	73.6	.4943	81.6
.4196	49.8	.4369	57.8	.4550	65.8	.4744	73.8	.4949	81.8
.4200	50.0	.4373	58.0	.4555	66.0	.4749	74.0	.4954	82.0
.4204	50.2	.4378	58.2	.4560	66.2	.4754	74.2	.4959	82.2
.4208	50.4	.4382	58.4	.4565	66.4	.4759	74.4	.4964	82.4
.4213	50.6	.4387	58.6	.4569	66.6	.4764	74.6	.4970	82.6
.4217	50.8	.4391	58.8	.4574	66.8	.4769	74.8	.4975	82.8
.4221	51.0	.4396	59.0	.4579	67.0	.4774	75.0	.4980	83.0
.4225	51.2	.4400	59.2	.4584	67.2	.4779	75.2	.4985	83.2
.4229	51.4	.4405	59.4	.4589	67.4	.4784	75.4	.4991	83.4
.4234	51.6	.4409	59.6	.4593	67.6	.4789	75.6	.4996	83.6
.4238	51.8	.4414	59.8	.4598	67.8	.4794	75.8	.5001	83.8
.4242	52.0	.4418	60.0	.4603	68.0	.4799	76.0	.5007	84.0
.4246	52.2	.4423	60.2	.4607	68.2	.4804	76.2	.5012	84.2
.4251	52.4	.4427	60.4	.4612	68.4	.4810	76.4	.5017	84.4
.4255	52.6	.4432	60.6	.4617	68.6	.4815	76.6	.5022	84.6
.4260	52.8	.4436	60.8	.4622	68.8	.4820	76.8	.5028	84.8
								.5033	85.0

pycnometer and convert the readings into percentage of sucrose by means of the Domke table (Sucrose from Apparent Specific Gravity), supplemented by the temperature correction table herewith. Correct for temperature, if other than 20°, in accordance with the second table. Although other solids besides sucrose are present, the method gives approximate results, since the individual sugars differ little in specific gravity.

In the *Methods of Analysis of the A.O.A.C.*, from which Domke's table is taken, Plato's

Sucrose Per Cent from Zeiss Immersion Refractometer Reading and Refractive Index (Schönrock-Mathews) *

Scale Reading 20° C. †	Ref. Index 20° C.	Sucrose Per Cent	Scale Reading 20° C.	Ref. Index 20° C.	Sucrose Per Cent	Scale Reading 20° C.	Ref. Index 20° C.	Sucrose Per Cent
14.47	1.33299	0.00	45	1.34463	7.91	76	1.35606	15.24
15	.33320	0.15	46	.34500	8.15	77	.35642	15.47
16	.33358	0.41	47	.34537	8.39	78	.35678	15.69
17	.33397	0.68	48	.34575	8.64	79	.35714	15.91
18	.33435	0.94	49	.34612	8.89	80	.35750	16.14
19	.33474	1.21	50	.34650	9.13	81	.35786	16.36
20	.33513	1.48	51	.34687	9.38	82	.35822	16.58
21	.33551	1.74	52	.34724	9.62	83	.35858	16.81
22	.33590	2.01	53	.34761	9.86	84	.35894	17.03
23	.33628	2.27	54	.34798	10.10	85	.35930	17.25
24	.33667	2.54	55	.34836	10.34	86	.35966	17.47
25	.33705	2.80	56	.34873	10.58	87	.36002	17.69
26	.33743	3.07	57	.34910	10.82	88	.36038	17.91
27	.33781	3.33	58	.34947	11.06	89	.36074	18.12
28	.33820	3.59	59	.34984	11.30	90	.36109	18.34
29	.33858	3.85	60	.35021	11.54	91	.36145	18.56
30	.33896	4.11	61	.35058	11.78	92	.36181	18.78
31	.33934	4.36	62	.35095	12.01	93	.36217	19.00
32	.33972	4.62	63	.35132	12.25	94	.36252	19.21
33	.34010	4.88	64	.35169	12.48	95	.36287	19.42
34	.34048	5.14	65	.35205	12.72	96	.36323	19.63
35	.34086	5.40	66	.35242	12.95	97	.36359	19.85
36	.34124	5.65	67	.35279	13.18	98	.36394	20.06
37	.34162	5.91	68	.35316	13.41	99	.36429	20.27
38	.34199	6.16	69	.35352	13.64	100	.36464	20.48
39	.34237	6.41	70	.35388	13.87	101	.36500	20.69
40	.34275	6.66	71	.35425	14.10	102	.36535	20.90
41	.34313	6.91	72	.35461	14.33	103	.36570	21.11
42	.34350	7.16	73	.35497	14.56	104	.36605	21.32
43	.34388	7.41	74	.35533	14.79	105	.36640	21.53
44	.34426	7.66	75	.35569	15.01			

* Schönrock: Landt: *Z. Ver. deut. Zucker-Ind.* 1933, **83**, 692; Mathews: *Methods of Analysis, A.O.A.C.*

† The scale readings are in Pulfrich units (*Z. angew. Chem.* 1899, p. 1168). Readings of other immersion refractometer scales must be converted into refractive indices before they can be used.

table is given to five places of decimals at 20°/20° and 20°/4° and degrees Baumé (Modulus 145) are added. In Browne and Zerban's *Sugar Analysis*, Domke's table is extended to five places of decimals and Plato's table to six places.

Correction of Sucrose Percentages by Abbé or Immersion Refractometer to 20°C (International Commission) *

Temperature °C.	Sucrose Per Cent										
	0	5	10	15	20	25	30	40	50	60	70
	Subtract from the per cent sucrose										
10	0.50	0.54	0.58	0.61	0.64	0.66	0.68	0.72	0.74	0.76	0.79
11	.46	.49	.53	.55	.58	.60	.62	.65	.67	.69	.71
12	.42	.45	.48	.50	.52	.54	.56	.58	.60	.61	.63
13	.37	.40	.42	.44	.46	.48	.49	.51	.53	.54	.55
14	.33	.35	.37	.39	.40	.41	.42	.44	.45	.46	.48
15	.27	.29	.31	.33	.34	.34	.35	.37	.38	.39	.40
16	.22	.24	.25	.26	.27	.28	.28	.30	.30	.31	.32
17	.17	.18	.19	.20	.21	.21	.21	.22	.23	.23	.24
18	.12	.13	.13	.14	.14	.14	.14	.15	.15	.16	.16
19	.06	.06	.06	.07	.07	.07	.07	.08	.08	.08	.08
	Add to the per cent sucrose										
21	0.06	0.07	0.07	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08
22	.13	.13	.14	.14	.15	.15	.15	.15	.16	.16	.16
23	.19	.20	.21	.22	.22	.23	.23	.23	.24	.24	.24
24	.26	.27	.28	.29	.30	.30	.31	.31	.31	.32	.32
25	.33	.35	.36	.37	.38	.38	.39	.40	.40	.40	.40
26	.40	.42	.43	.44	.45	.46	.47	.48	.48	.48	.48
27	.48	.50	.52	.53	.54	.55	.55	.56	.56	.56	.56
28	.56	.57	.60	.61	.62	.63	.63	.64	.64	.64	.64
29	.64	.66	.68	.69	.71	.72	.72	.73	.73	.73	.73
30	.72	.74	.77	.78	.79	.80	.80	.81	.81	.81	.81

* *Intern. Sugar J.* 1937, **39,** 24s, via *Methods of Analysis, A.O.A.C.*

Refractometric Method. Although not so exact as direct determination by a standard method, calculation from the refractometer reading has a wide application in the food laboratory. It is more accurate and requires less labor than calculation from the specific gravity, although requiring more expensive apparatus. The common sugars (sucrose, dextrose, levulose, maltose, and lactose) have practically the same refractive index and dextrin, although having a somewhat higher index, does not appear to affect seriously the results on materials likely to be examined.[33]

Process. The manipulation is exceed-

ingly simple. Smear a film on the prism surface of the Abbé instrument or dip the prism itself of the immersion instrument into the liquid, adjusted to 20° (Schönrock) or 28° (Geerlig), and take the reading. The Geerlig temperature is convenient for tropical laboratories. Note carefully the temperature, if other than standard.

It is imperative that the solution be clear and not too dark. Dilution with a definite amount of a concentrated colorless sugar solution of known refraction is recommended in the Official Method of the A.O.A.C., if the color is too dark for reading.

Calculation. Refractometers with a sugar scale, like saccharimeters, give the percentage directly. Convert readings in terms of refractive indices on the Abbé scale into corresponding sucrose percentages by using the International Commission-A.O.A.C. table and those in terms of the readings of the immersion refractometer scale by using the Schönrock-Mathews table. Correct for temperature in accordance with the National Bureau of Standards table based on Plato's data, all given herewith.

Protein

Determine the small amount of nitrogen by the Kjeldahl or Kjeldahl-Gunning method and calculate the protein, using the factor 6.25.

Fat

Only traces of fat, at most, are present in sugar house products. See Part II, E7, below.

Sucrose; Invert Sugar

See Part II, E2, Sucrose, Invert Sugar; E2, Sucrose (Density and Refractometric Methods for Solids).

Sucrose and Raffinose

Sugar beet juices and crude products contain raffinose in amounts sufficient to vitiate the results by the Clerget method or its modifications. When the direct reading of a sample shows a higher percentage of sucrose than that calculated by any of the foregoing formulas, the presence of raffinose is indicated and a special calculation formula must be applied.

Creydt-Browne and Gamble Polarimetric Method.[34] The method, so far as the process is concerned, is the same as those designed for sucrose alone; it is the details of calculation that are markedly different from the calculation methods devised by Clerget, Herzfeld, and Schrefeld. The formulas proposed by Creydt remained unchallenged until Browne pointed out certain errors and added to the revised formulas others applicable at all temperatures here given. The manipulation, which follows, is that recommended by Schrefeld.

Process. *Direct Polarization.* Proceed as in the Clerget-Schrefeld Method above, taking the reading at 20°.

Invert Polarization. Measure 50 ml. of the cleared solution into a 100-ml. flask, add 25 ml. of water and 5 ml. of *hydrochloric acid* (sp.gr. 1.19), and close with a rubber stopper carrying a thermometer, the bulb of which is covered by the liquid. Immerse in a large water bath kept at 70°. When the thermometer in the flask registers 67°, which requires about 3 minutes, continue the heating 5 additional minutes, the temperature at the end being about 69.5°. Cool quickly, rinse thermometer, make up to 100 ml. at 20°, mix, polarize in a 400-mm. tube, and substitute the reading directly in the formula or polarize in a 200-mm. tube and multiply the reading by 2.

Calculation. Calculate by Browne's formulas as follows, those designated I being for readings at 20°, those designated II for readings at all temperatures:

I 0.844 $R = \frac{0.33(P + P')}{1.563}$

$$\frac{P(0.478 \times 0.0018t') - P(1.006 - 0.0003t)}{(0.908 - 0.0032t')(1.006 - 0.0003t)}$$

II

$$\frac{.006 - 0.0003t)}{(1.681 - 0.0059t')(1.006 - 0.0003t)}$$

in which S is per cent of sucrose, R is per cent of anhydrous raffinose, P is direct reading, P' is invert reading, t is the temperature of the direct polarization, and t' is the temperature of the invert polarization.

When all the temperatures are 20°, formulas I become the same as formulas II.

Paine and Balch Two Enzyme Inversion Polarimetric Method.[35] This method overcomes the errors due to acid hydrolysis in the determination of sucrose and raffinose, as does the invertase inversion method in the determination of sucrose above. Directions are given for the preparation of the enzyme solutions and the procedure, also calculation formulas. The application of the method is chiefly in the sugar beet industry.

Ash

See Part I, C2*f*, C8*a*, Part II, E2.

Lorge, Sattler, and Zerban Conductivity Calculation Method.[36] The following formula was developed at the New York Sugar Trade Laboratory [37] by Sattler and Zerban and by Zerban and Sattler for the determination of the per cent of ash (A) in raw and refinery sirup and in molasses:

$$(1)\quad A = 0.019137K - 0.002249K_2 - 0.00121K_3 + 3.07$$

in which K is the specific conductance $\times 10^6$ of the solution of 0.5 g. of the sample and 4.5 g. of pure sucrose, diluted to 100 ml., K_2 is that of the solution made by adding 5 ml. of 0.25 N potassium hydroxide solution to 200 ml. of the above solution, and K_3 is that of a solution made by adding 5 ml. of 1.0 N orthophosphoric acid to 200 ml. of the above solution. The addition of the sucrose was to simulate a raw cane sugar solution, and thus relate the analysis of sirup and molasses directly with the method for ash conductivity in raw cane sugar.

In this paper Lorge, Sattler, and Zerban advocate omission of the sucrose for routine work and propose the following formula:

$$(2)\quad A = 0.01556K - 0.001125K_1 - 0.000623K_2 - 0.000219K_3 + 3.083$$

in which K, K_2, and K_3 are the same as in (1) and K_1 represents the specific conductance $\times 10^6$ of the solution obtained by adding 5 ml. of 0.25 N hydrochloric acid to 200 ml. of the solution of the product as specified above.

The second formula applies in the presence of hydrochloric acid, phosphoric acid, and potassium hydroxide. It was developed, on the basis of the statistical technique of multiple regression, out of the interrelations existing among the conductance values corresponding to the content of ash by chemical analysis. The error is only 3 parts in 93, whereas 3 parts in 50 is allowed for the chemical method.

Glucose

Because of the variable composition and physical values, only an approximate calculation of the amount of glucose present in mixtures with molasses or sugar house sirup, maple sirup, or honey is possible.

Modified Leach Polarimetric Calculation Method.[38] At the time Leach suggested his original calculation formula, 42° Baumé glucose polarized 175° V. at ordinary temperature and 163° V. at 87° (the temperature at which invert sugar polarizes 0° V.); later he announced that these figures no longer were valid, owing to changes in composition of the commercial product.

These are the Official A.O.A.C. formulas:

$$\frac{(P - S)100}{211} \qquad G' = \frac{200P'}{196}$$

in which G and G' are percentages of glucose

solids polarizing +211° V. in substances containing little or no invert sugar and containing invert sugar respectively, P is direct polarization, P' is polarization at 87° in a 200-mm. tube of 50 ml. of a deleaded half-normal solution, diluted with 25 ml. of water, inverted at 67° with 10 ml. of HCl (sp.gr. 1.1029), cooled, nearly neutralized to phenolphthalein, cleared with alumina cream, and made up to 100 ml.

4. COMMERCIAL GLUCOSE

Glucose, mixing glucose, or confectioner's glucose—not to be confused with the sugar *d*-glucose or dextrose—according to the U. S. Standards, ranges in density from 41 to 45° Baumé at 100° F. (37.7° C.). No polarization figures are given in the Standards, but the product must vary greatly if not in the ratio of 41 to 45. It is defined as being "made by incompletely hydrolyzing starch or a starch-containing substance."

The composition during the successive stages of conversion of starch, as indicated by the change in color with iodine solution from blue, through purple, red, and yellow to colorless, is a continually changing mixture of two or more intermediate products—soluble starch, amylodextrin, erythrodextrin, achroodextrin, maltodextrin (probably other dextrins), maltose, and finally dextrose. Interpretation of quantitative data, notably copper reduction, polarization readings and water content, involves complicated formulas of questionable value. Fortunately the practical problem is usually simple, such as determination of the end-point of the hydrolysis or the compliance with specifications of a standard or a contract.

Water

Cleland and Fetzer Filter-Cel Vacuum Oven Gravimetric Method.[40] Three procedures, developed in the laboratory of the Union Starch and Refining Co., are given: (1) referee weighing bottle modification, (2) routine dish modification, and (3) vacuum flask modification.

I. Referee Weighing Bottle Modification.[41] Apparatus. *Weighing Bottle*, 250 ml., glass stopper.

Pestle, detachable, consisting of a Pyrex test tube in which is inserted a glass rod made fast by two rings.

Vacuum Oven.

Individual Desiccators, 5- to 10-pound friction-top cans with phosphorus pentoxide as desiccant.

Reagent. *Filter-Cel.* See Cleland and Fetzer Modification of the Bidwell and Sterling Toluene Distillation Volumetric Method, Part II, A1.

Process. *Charge.* Weigh 25 g. of *Filter-Cel* into duplicate weighing bottles, introduce a pestle in each, place in a vacuum oven with the stopper set at a 90° angle in the mouth, together with an empty bottle used as a tare, and dry to constant weight. Pipet into one of the bottles a quantity of the sample containing 5 to 8 g. of solids, or if the sample is below 35° Baumé, weigh, run 20 to 40 ml. of the solution on the Filter-Cel, stopper, and reweigh; if over 35° Baumé, run 8 to 10 g. on a nickel scoop to which 10 ml. of water are added, warm, and deliver onto the Filter-Cel, and wash with three 5-ml. portions of water. In either case gently work up into a damp mass with a detachable pestle, leaving the test tube in the flask. The ratio of Filter-Cel to sample must be such as to yield a damp mass so that the Filter-Cel retains its powdery form.

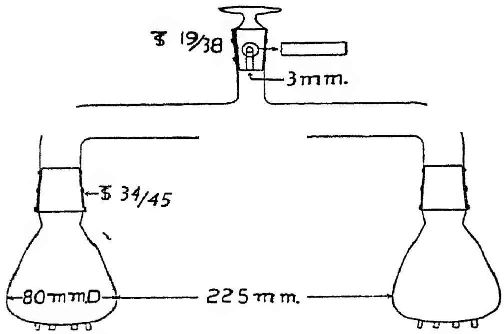
Preliminary Drying. Dry in the vacuum oven under a pressure of 50 to 75 mm. by a water pump for 2 to 4 hours. Rework the Filter Cel mass to a powder.

Final Drying. Continue the drying using a Hyvac or Megavac pump, reworking to a powder at an early stage. Complete the drying (about 15 hours) to constant weight, that is, within 1 mg. or 0.01%.

II. Routine Dish Modification. Use aluminum dishes 25 mm. high and 75 mm. in diameter and dry in the vacuum oven at 100°, reworking as above described.

III. Vacuum Flask Modification. This procedure, employing apparatus essentially as devised by Lobry de Bruyn and Van Laent, dispenses with the vacuum oven in the final drying.

APPARATUS. An *Assembly* (Fig. 140) of two flasks joined through taper joints with a curved tube connected with a Hyvac pump.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1941, 13, 859

FIG. 140. Cleland and Fetzer Moisture Assembly.

A *Train* consisting of (*a*) two 19-liter (5-gal.) bottles containing about 1.25 cm. of sulfuric acid, (*b*) a sulfuric acid tower fitted with a fluted diffuser, (*c*) a tower with cotton, (*d*) a tower with Drierite, and (*e*) a large vessel fitted with a trap covered with phosphorus pentoxide.

REAGENTS. *Filter-Cel.*

Phosphorus Pentoxide.

PROCESS. *A. Oven Drying.* Place 10 g. of *Filter-Cel* in one of the two flasks, weigh 5 g. of sirup in a nickel scoop, dilute with 5 ml. of water, and pour onto the Filter-Cel, rinsing with three 2-ml. portions of water and mixing well. Place the flask in a vacuum oven at 38° connected through a receiver containing a large quantity of calcium chloride, with a Hyvac pump and let stand overnight.

B. Flask Drying. Grind the mass to a fine powder with a Pyrex test tube inserted in the flask. Place in the second flask *phosphorus pentoxide*, lubricate the joints with a small amount of *Cello grease*, and assemble the parts. Exhaust the system, close the cock, and disconnect the pump. Place the flask containing the Filter-Cel in an air bath at 37 to 38° and the other flask in a water bath of running water to maintain a temperature drop.

Check the vacuum daily by a McLeod gage and repump if necessary. Rotate the flasks daily to expose a fresh surface.

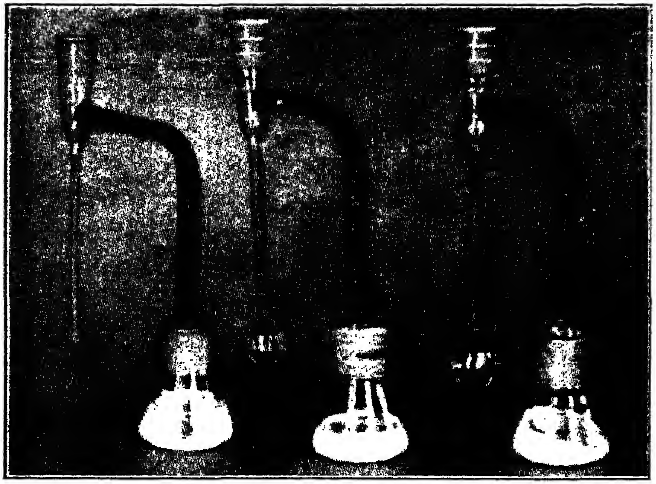
Weighing. Before disconnecting for weighing, introduce air through the drying train, then stopper, and weigh.

Nearly constant weight is reached in 4 days, but a further loss of 0.20% in the author's determination was sustained during 4 additional days. Practically the same results on corn sirup were obtained at 100° as at 37 to 38°.

Bidwell and Sterling Toluene Distillation Method. As originally devised at the U. S. Bureau of Chemistry (Part II, A1), the method was applied to a wide variety of foods, but as adopted as an Official Method it is limited to grain and stock foods. Rice,[42] of the National Sugar Refinery, in applying the method to sugar sirup, made the important addition of Filter-Cel.

Cleland and Fetzer Filter-Cel Benzene Distillation Method.[43] The method (Union Starch and Refining Co.) meets the demand for a more accurate means of determining water in corn sugar (70 or 80°). It also has a wider application.

APPARATUS. *Distillation Assembly* (Fig. 141), consisting of a 250-ml. Erlenmeyer flask joined to a bulb-trap by a taper ground joint. By adding 15- to 30-ml. bulbs, the capacity of the Bidwell and Sterling trap was increased correspondingly, without increasing the diameter of the graduated portion with attending loss in accuracy.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1942, **14,** *126*

FIG. 141. Cleland and Fetzer Distillation Assembly.

REAGENT. *Filter-Cel.* Wash with dilute HCl, then with water until neutral, and dry at 105°.

PROCESS. Weigh 30 g. of washed *Filter-Cel* into the flask and dry to constant weight at 100° in a vacuum oven (4 hours). Add a suitable amount of the sample (regulated according to the moisture content and the size of the trap) and reweigh to obtain the weight of the charge. Run into the flask 100 ml. of *benzene* and work into a homogeneous mass by means of a glass rod attached to the neck of the flask by a short length of thin-walled rubber tubing, thus protecting against evaporation. Connect with the trap and condenser and heat gradually so that 2 drops distil per second. After 1 hour, during which 90% of the water is removed, increase the rate to 4 drops per second. When all water is driven over, cool the trap to 20° and read the volume.

5. MAPLE PRODUCTS

General Composition. The range in percentage of food constituents of maple sirup and maple sugar is given below.[44]

	Maple Sirup (481 Samples, as Sold)					Maple Sugar (363 Samples, Dry Basis)			
	Water	Sucrose	Invert sugar	Ash	Undetermined	Sucrose	Invert sugar	Ash	Undetermined
	%	%	%	%	%	%	%	%	%
Minimum	24.85	47.20	0.00	0.46	0.00	57.04	0.09	0.76	0.00
Maximum	48.14	70.46	11.01	1.06	4.51	98.62	37.30	1.70	8.18
Average	**34.22**	**62.57**	**1.47**	**0.66**	**1.08**	**90.69**	**6.19**	**0.98**	**2.14**

In the table below are given percentages and values of use in judging the purity of the product.[44]

	Maple Sirup (481 Samples)					Maple Sugar (363 Samples)				
	Ash			Winton lead No.	Malic acid No.	Ash			Winton lead No.	Malic acid No.
	Total	Sol.	Insol.			Total	Sol.	Insol.		
	%	%	%			%	%	%		
Minimum	0.68	0.35	0.23	1.76	0.21	0.76	0.31	0.21	1.85	0.51
Maximum	1.68	1.23	1.01	4.41	1.82	1.70	1.14	1.00	4.95	1.72

Sample

For determination of moisture, grind and mix well maple sugar; for determination of solids in clear maple sirup, use the sample in its original condition. If crystals are present in the sirup, heat at 50° in a closed vessel until dissolved, straining if necessary.

For other determinations, acting on the advice of Jones, chemist of the Vermont Experiment Station, Bryan[45] recommends using a clear sirup containing 65% of solids, prepared by dissolving maple sugar or adjusting maple sirup to that density. By so doing, insoluble organic and mineral constituents are eliminated which otherwise would permit dilution with sugar sirup without reduction of the ash and other characteristic constituents below legal standards.

In the Methods of Analysis of the A.O.A.C. it is directed to dilute maple sirup to 64.5% of solids, as indicated by the refractive index 1.4521, or other convenient value, then filter so as to secure 100 ml. in 5 minutes and adjust to exactly 65%.

Electrical Conductivity Number

Snell Method.[46] Apparatus. *Electric Current* of 110 volts and galvanometer or *Dry or Storage Cell* with microphone hummer (or induction coil) and telephone. The resistances of 10 and 100 ohms should be fixed and accurate.

Conductivity Cell. A low voltage cell of resistance glass, with a constant of about 0.15, provided with a thermometer graduated in tenths from 20 to 30°. The platinized platinum electrodes are either sealed into the vessel used for the solution of the sample (Zerban type) or attached to a support for lowering into the solution. A control device holds the temperature at 25° ± 1°.

Determine the cell constant by measuring the electrical resistance at 25° of 0.01 and 0.02 *M* solutions of potassium chloride and multiply the number of ohms by 141.2 and 276.1 respectively, then average the results.

Slide Wire or *Wheatstone Bridge.*

Process. Weigh into a 100-ml. volumetric flask a quantity of the sirup containing 25 g. of dry matter, dissolve in warm water from the same lot as used in determining the cell constant. Cool to 25° and adjust to the mark. Measure the resistance at 25° ± 1° and divide the cell constant by the number of ohms thus found.

Solids (Water); Reducing Sugars; Sucrose; Commercial Glucose

See Part II, E2, E3, and E4.

Lead Number

A copious precipitate formed by basic lead acetate in clearing a solution of maple sugar or diluted maple sirup for polarization has long been regarded as a rough indication of purity, since sugar sirups colored with caramel in imitation of the genuine product give little or no precipitate with the reagent. The precipitate consists of lead salts of organic (chiefly malic) and inorganic acids and other constituents.

Jones [47] went a step further and developed, for the use of farmers and wholesale buyers, a quantitative method in which the volume of the precipitate separated in the lower end of a graduated tube is measured by centrifugal force. About the same time Hortvet [48] devised, for the use of food inspectors, a similar method which was for a time a provisional method of the A.O.A.C. Two years later Sy [49] described a gravimetric method in which the lead in the precipitate is determined. A month before Sy's paper was published, Winton and Kreider presented a paper at the Summer Meeting of the American Chemical Society giving results by an indirect gravimetric method devised by one of us who also suggested the name *lead number* for the grams of lead in the precipitate from 100 g. of the product, analogous to the iodine number of fats and oils. McGill [50] in 1911 described a gravimetric method in which the lead complex is collected on a Gooch crucible and weighed, and Snell, MacFarlane, and Van Zoeren [51] in 1916 a volumetric method, plotting the electrical resistance found against the volumes of basic lead acetate solution added.

Winton Indirect Lead Subacetate Gravimetric Method.[52] Reagent. *Standard Lead Subacetate Solution.* Dilute the reagent designed for clearing cane and beet products (Part II, E2) with 4 volumes of boiled water, filtering if not clear, or prepare as described for the McGill Method below.

Process. *Lead Precipitation.* Weigh 25 g. of the sample in a beaker or sugar dish and wash into a 100-ml. volumetric flask with boiled water. If desired, 26 g. may be weighed so as to permit polarization on the same solution. To the solution, add 25 ml. of *standard lead subacetate solution*, fill to the mark, shake, allow to settle 3 hours, and filter through a dry paper.

Determination of Excess of Lead. Pipet 10 ml. of the filtrate into a beaker, dilute to about 50 ml., add a moderate excess of *sulfuric acid* and 100 ml. of *ethanol*. After standing overnight, collect the lead sulfate on a Gooch crucible, wash with *ethanol*, dry cautiously, ignite for 3 minutes at low redness, avoiding the reducing flame, cool, and weigh.

Blank. Carry on at the same time as the actual analysis a blank determination on 25 ml. of the *standard lead subacetate*, acidified with a few drops of *acetic acid* as recommended by A. H. Bryan, proceeding in other details as above described.

Calculation. Obtain the Winton lead number (L), which is the weight of lead in the precipitate from 100 g. of the sample, by the following formula:

$$\frac{(B - A) \times 0.6833 \times 100}{2.5}$$

$$27.33 \qquad - A)$$

in which B and A are the weights of lead sulfate found in the blank and actual determination respectively, 0.6833 is the conversion factor of lead sulfate to lead, and 2.5 is the grams of the sample in the aliquot.

McGill Canadian Lead Number Gravimetric Method.[53] Reagent. *Lead Subacetate Solution.* Fowler [54] prepares the solution as follows.

Heat PbO at 650 to 670° in a muffle for 2.5 to 3 hours. Weigh 40 g. of the lemon-yellow activated litharge thus obtained and 80 g. of $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ into an Erlenmeyer flask, add 250 ml. of water, and reflux for 45 minutes. After cooling, filter and dilute the

filtrate with boiled water to sp.gr. 1.25 at 20°.

PROCESS. *Lead Precipitation.* Weigh a portion of the sugar, or a sirup made from it, representing 25 g. of dry matter, into a 100-ml. volumetric flask and dilute to the mark at 20°, or employ for the determination the solution in which the conductivity number was determined. Pipet 20 ml. into a large test tube, precipitate with 2 ml. of *lead subacetate solution*, mix, cork, and allow to settle for 2 hours.

Filtration and Drying of Lead Precipitate. Collect the precipitate on a 25-ml. tared Gooch crucible with an asbestos mat at least 3 mm. thick and wash with cold water 4 times, taking care to avoid excessive suction and a complete removal of the liquid with the formation of fissures. Dry at 100° and weigh.

CALCULATION. Calculate the *Canadian lead number*, which is the weight of lead precipitate (not metallic lead), by multiplying the weight of the precipitate by 20.

MALIC ACID NUMBER

Leach and Lythgoe-Cowles Calcium Malate Volumetric Method.[55] REAGENT. *Calcium Acetate Solution.* Dissolve 10 g. of calcium acetate in water and make up to 100 ml. Leach and Lythgoe used calcium chloride solution in conjunction with ammonium hydroxide.

PROCESS. *Calcium Malate Precipitation.* Tare a 150-ml. lipped beaker and weigh into it 6.7 g. of the sample. Dilute with 5 ml. of water, add 2 ml. of *calcium acetate solution*, then with stirring 100 ml. of *ethanol*, and warm until the precipitate settles and the supernatant liquid is clear. Collect the precipitate on a filter, wash with 75 ml. of *85% ethanol*, dry, and ignite in a platinum dish.

Titration. Add 10 ml. of *0.1 N hydrochloric acid*, warm slightly until all the lime dissolves, cool, and titrate back with *0.1 N sodium hydroxide solution*, using *methyl orange* as indicator.

Blank. Make a blank determination, using the same amount of the reagents as in the actual analysis.

CALCULATION. Divide the number of milliliters of 0.1 *N* acid less the number of milliliters of 0.1 *N* alkali by 10 to obtain the malic acid number.

ASH

See Part I, C2*f*, and Part II, E2, for methods of determination of ash, soluble ash, and alkalinity of ash.

LEAD

Fischer Dithizone Colorimetric Method. See Part I, C8*b*.

Perlman[56] of the New York State Food Laboratory, Albany, employs a field technique similar to that furnished in mimeographed form by the U. S. Food and Drug Admin., Sept. 17, 1933.

Roberge and Landry Dithizone-Electrodeposition Iodometric Method.[57] The steps are (1) treatment with *nitric acid*, (2) extraction of lead with *diphenylthiocarbazone*, (3) electrical deposition, and (4) titration with *potassium iodide* and *sodium thiosulfate*.

Notwithstanding the several reactions involved, the method is exceptionally rapid (60 determinations per day) and accurate.

ZINC

Fischer Dithizone Iodometric Method. See Part I, C8*b*, Zinc.

Perlman and Mensching Modification.[58] PROCESS. *Solution.* Place 25 g. of a sirup, or a sugar reconstituted into a sirup, in a 500-ml. tall-form beaker. If less than 0.1 mg. of zinc is present, use 50 g. Add 50 ml. of water and 10 ml. of *zinc-free nitric acid*, mix, cover, and boil for 15 to 20 minutes.

Buffer Addition. Transfer the solution to a 500-ml. separatory funnel, dilute to 350 to

400 ml., add 20 ml. of *25% citric*
and neutralize to *litmus* with *ammonium hydroxide*, avoiding more than 0.5 ml. excess.

Dithizone Treatment. Cool the solution, add 15 ml. of *0.2% dithizone solution in chloroform* and shake vigorously until the reagent no longer changes color. Draw off the chloroform layer into a 125-ml. separatory funnel containing 20 ml. of *1 + 999 ammonium hydroxide*. If the chloroform solution is red as it runs down the funnel stem, reextract with another 15-ml. portion of the *0.2% dithizone solution*, repeating until the color is blue or purple, then continue the extraction, using *0.1* and finally *0.01% strength* until consecutive extracts are green in color. Test the original solution between extractions and add a drop or two of *strong ammonium hydroxide* if necessary.

Ammonia Extraction. Shake the combined extracts with *dilute ammonium hydroxide*, allow to separate, swirl to dislodge adhering extract, and draw off the dithizone-chloroform layer, together with a small amount of emulsion, into a 125-ml. separatory funnel containing exactly 25 ml. of *1% nitric acid*.

Acid Extraction. Shake the dithizone extract vigorously with the dilute acid until the original green color is restored and continue with the shaking for 3 to 4 minutes. Draw off the dithizone-chloroform extract and reserve for recovery of the chloroform.[59] Filter the acid layer through a small dry paper, pipet 20 ml. of the clear filtrate into a dry 200-ml. tall-form beaker, and evaporate to dryness on the water bath. Add to the dry residue 2 to 3 ml. of *ethanol* and cautiously bring to a boil. When the maximum amount of organic matter has dissolved, add slowly 5 ml. of *0.02 N sulfuric acid*, washing down the sides. Again bring to a boil, boil for 1 minute with swirling, add 10 ml. of water, boil gently for 3 to 5 minutes, then cool to room temperature.

Titration. Add 1 ml. of *0.5% starch solution* and 1 ml. of fresh *10% potassium iodide solution*, mix, and let stand a minute. If, owing to other metals, iodine is liberated, add dropwise *0.001 N sodium thiosulfate solution* until the starch-iodide color is discharged. Add 1 ml. of freshly prepared *potassium ferricyanide solution*, stir, let stand 3 minutes, and titrate the liberated iodine against the *thiosulfate solution* until a drop causes no change in the yellow color at the point of contact. If more than 10 ml. of thiosulfate solution is required, repeat the determination, using a smaller charge or an aliquot of the sulfuric acid solution of the preceding paragraph.

CALCULATION. Use the formula: 1 ml. of 0.001 N sodium thiosulfate solution = 0.1 mg. of zinc. Correct for the thiosulfate required in a blank determination.
the milliliters of thiosulfate required by
conversion factor, then by 0.1 and 1000;
divide the product by the grams of the sample represented by the portion titrated. The result is the gammas per gram of zinc in the sample.

NOTE. Lead, tin, copper, bismuth, and thallium do not interfere.

6. HONEY

Microscopic Examination. By diluting honey, centrifuging, and examining the sediment under the microscope, some idea as to its source may be gained from the pollen grains present. Bees visit flowers both to gather nectar and to obtain pollen for food. Many of the pollen grains find their way into the finished honey. When there is a great preponderance of pollen from one species (e.g., clover, sage, orange blossom), it is safe to claim that particular flower as the source, but usually many forms are represented in a single honey and the analyst must have a wide acquaintance with the pollen of many species.

General Composition. The following table by Browne[60] shows the composition and polariscopic data on 92 samples of levorota-

tory honey and 7 samples of dextrorotatory honey. The latter were made by bees from the inflorescence of certain species such as white oak and poplar and from honeydew.

	Water	Invert Sugar	Sucrose	Dextrin	Acid as Formic	Ash	Polarization				
							Direct			Invert	
							Immediate 20° C.	Constant 20° C.	87° C.	20° C.	87° C.
	%	%	%	%	%	%	°V.	°V.	°V.	°V.	°V.
Levorotatory											
Minimum	12.42	62.23	0.00	0.04	0.04	0.03	−21.9	−24.8	+0.5	−29.3	−0.7
Maximum	26.88	83.36	10.01	7.58	0.25	0.90	+3.7	−0.3	+23.7	−1.3	+23.2
Average	**17.70**	**74.98**	**1.90**	**1.51**	**0.08**	**0.18**	**−11.2**	**−14.7**	**+10.2**	**−19.2**	**+7.9**
Dextrorotatory											
Minimum	13.56	64.84	0.61	6.02	0.05	0.29	+6.7	+3.6	+28.5	−2.5	+20.9
Maximum	17.80	71.69	5.28	12.95	0.19	1.29	+24.9	+17.8	+35.8	+15.0	+35.0
Average	**16.09**	**66.96**	**3.01**	**9.70**	**0.12**	**0.81**	**+14.8**	**+9.4**	**+32.2**	**+5.5**	**+27.6**

Analytical Methods. Browne's monograph, issued in 1908, was so exhaustive that few have entered the field since. Practically all the methods described during 1908 or earlier are those which had his stamp of approval (whether or not so qualified) and are now A.O.A.C. methods.

Sample

Remove the caps of comb honey and strain through a fine sieve or cheese cloth. Strained honey, if clear, needs no preparation. If either comb or strained honey has granulated, heat at 50° in a closed vessel until in complete solution.

Water

Determine by the sand method (Part II, E3), drying at 65 to 70° in a vacuum oven, employing 2 g. of the sample, or an aliquot representing that quantity, and 10 g. of sand. Moisten with water to distribute the honey through the sand. According to Browne,[61] ordinary honey dries in about 12 hours, but honeydew honey, because of the presence of dextrin and gum, may require more than 36 hours.

Chataway Time-of-Fall Viscometric Method.[62] In addition to devising a method depending on the time-of-fall of a steel ball through a definite height of the honey column, Chataway (National Research Laboratories, Ottawa) prepared a table from his own data showing the relationship between (1) the percentage of moisture by the A.O.A.C. Method, (2) the viscosity as measured by the time-of-fall of a large and small ball through a 14-cm. column, and (3) the refractive index at 25°. The viscosity method has the advantage of employing apparatus of negligible cost readily assembled in any laboratory; the temperature, however, must be read with great exactness.

Apparatus. *Cylinder of Glass Tubing,* about 25 cm. long and 15 mm. inside diameter, with file marks showing a distance of 14 cm., firmly fixed in an accurately vertical position. This small tube permits a determination on a 3-ounce sample; when a sample of sufficient quantity is available, an ordinary measuring cylinder may be used. A

thermometer, graduated to 0.1°, is so clamped as to be in the center of the tube and about 1 inch below the surface of the honey.

Steel Ball-Bearing Balls, "S.K.F." $\frac{3}{16}$ inch (about 5 mm.) and $\frac{3}{32}$ inch (about 2.5 mm.) in diameter, for thick and thin honey respectively. The large balls weigh 0.4370 ± 0.0002 g.

Stop-Watch.

PROCESS. Fill the cylinder and allow the honey and the ball to acquire the same temperature, which should be as near 25° as possible. Extreme accuracy in measuring the temperature is more essential than in measuring the time of fall. In the case of thick honey, drop the *ball* down between the thermometer and the wall, then by tilting the cylinder bring it into the middle of the tube; in the case of thin honey, take the temperature and remove the thermometer before dropping the ball. Note the time and temperature when the ball reaches the 0 mark and again when it reaches the 14-cm. mark.

CALCULATION. Multiply the reading by the proper temperature correction factor (f) as found in the first table below and obtain the corresponding percentage of moisture from the second table. Chataway's graph

ABRIDGED TABLE * OF CORRECTION FACTORS (f) FOR VISCOSITY MEASUREMENTS (CHATAWAY)

Time of Fall	Temperature										
	24.0°	24.2°	24.4°	24.6°	24.8°	25.0°	25.2°	25.4°	25.6°	25.8°	26.0°
sec.	f	f	f	f	f	f	f	f	f	f	f
2.5							1.022	1.045	1.070	1.080	1.110
5.0	0.894	0.915	0.935	0.955	0.977	1.000	.023	.046	.072	.088	.113
7.5	.892	.913	.933	.955	.976	.000	.024	.047	.073	.093	.119
10.0	.889	.911	.932	.954	.976	.000	.024	.048	.075	.098	.124
12.5	.887	.909	.931	.953	.975	.000	.025	.049	.076	.101	.128
15.0	.885	.907	.930	.952	.975	.000	.025	.050	.078	.103	.132
17.5	.883	.906	.928	.951	.975	.000	.026	.051	.079	.106	.135
20.0	.882	.904	.927	.950	.974	.000	.026	.051	.080	.108	.137
25.0	.878	.901	.926	.949	.974	.000	.027	.053	.082	.111	.142
30.0	.876	.899	.924	.948	.973	.000	.027	.054	.083	.114	.145
35.0	.874	.897	.922	.947	.973	.000	.028	.055	.085	.116	.148
40.0	.871	.895	.921	.946	.972	.000	.028	.056	.086	.118	.150
50.0	.868	.892	.918	.944	.971	.000	.029	.057	.088	.121	.153
60.0	.866	.889	.916	.943	.971	.000	.030	.058	.090	.123	.156
70.0	.863	.888	.915	.942	.970	.000	.030	.060	.092	.124	.158
80.0	.862	.886	.914	.941	.970	.000	.031	.061	.092	.126	.160
90.0	.860	.885	.913	.940	.970	.000	.031	.062	.093	.126	.161
100.0	.859	.885	.912	.940	.969	.000	.031	.062	.093	.127	.161
110.0	.858	.884	.911	.939	.969	.000	.032	.063	.094	.127	.161
120.0	.857	.884	.910	.939	.969	.000	.032	.063	.094	.127	.161
140.0	.856	.883	.910	.938	.970	.000	.032	.063	.093	.126	.159
160.0	.856	.883	.910	.938	.970	.000	.031	.062	.092	.124	.157
180.0	.856	.883	.909	.938	.970	.000	.030	.062	.092	.122	.153
200.0	.856	.884	.908	.938	.970	.000	.029	.060	.089	.119	.148
240.0	.857	.884	.909	.938	.970	.000	.026	.057	.085	.113	.137

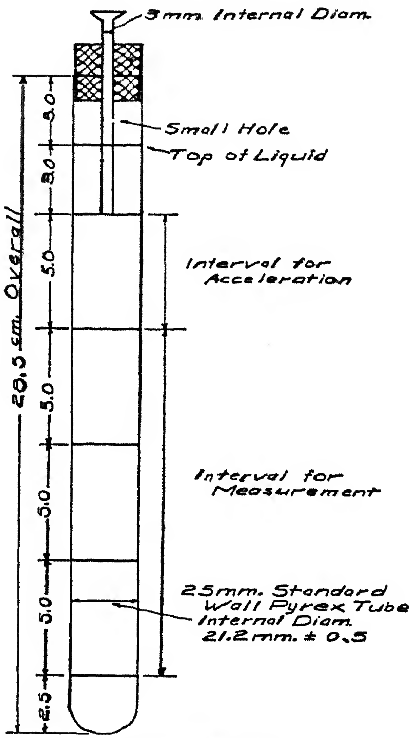
* The original three-page table covers temperatures from 15 to 30° and time-of-fall up to 1400 seconds. If the abridged table is used, the greatest care must be taken in regulating and reading the temperature.

Calculation of Moisture from Viscosity * and Refraction † (Chataway)

Water	Viscosity 25° Large Ball	Refractive Index 25°	Water	Viscosity 25° Large Ball	Refractive Index 25°	Water	Viscosity 25°		Refractive Index 25°
							Large ball	Small ball	
%	sec.	n	%	sec.	n	%	sec.	sec.	n
12.0	982	1.50550	15.4	102.8	1.49681	18.8	19.86	47.5	1.48840
.1	906	.50529	.5	97.5	.49657	.9	19.05	45.6	.48811
.2	840	.50504	.6	92.1	.49632	19.0	18.28	43.7	.48786
.3	776	.50481	.7	87.5	.49607	.1	17.54	42.0	.48762
.4	719	.50454	.8	83.2	.49581	.2	16.79	40.2	.48732
.5	668	.50427	.9	79.1	.49557	.3	16.07	38.5	.48713
.6	618	.50401	16.0	75.0	.49533	.4	15.45	37.0	.48690
.7	575	.50374	.1	70.8	.49507	.5	14.83	35.5	.48666
.8	535	.50346	.2	67.4	.49482	.6	14.22	34.0	.48641
.9	496	.50319	.3	63.6	.49457	.7	13.65	32.7	.48618
13.0	462	.50293	.4	60.7	.49431	.8	13.15	31.5	.48593
.1	428	.50266	.5	57.8	.49405	.9	12.65	30.3	.48570
.2	400	.50240	.6	54.8	.49382	20.0	12.16	29.1	.48548
.3	374	.50219	.7	52.1	.49357	.1	11.70	28.0	.48526
.4	348	.50189	.8	50.0	.49333	.2	11.25	26.9	.48503
.5	325	.50162	.9	47.5	.49307	.3	10.84	25.9	.48481
.6	304	.50136	17.0	45.4	.49283	.4	10.45	25.0	.48460
.7	285	.50111	.1	43.3	.49259	.5	10.07	24.1	.48437
.8	266	.50084	.2	41.2	.49234	.6	9.68	23.2	.48416
.9	251	.50061	.3	39.1	.49210	.7	9.33	22.3	.48394
14.0	237.7	.50035	.4	37.1	.49185	.8	9.03	21.6	.48373
.1	221.3	.50010	.5	35.5	.49160	.9	8.69	20.8	.48352
.2	208.4	1.49985	.6	33.7	.49134	21.0	8.38	20.1	.48329
.3	196.3	.49960	.7	32.2	.49108	.1	8.07	19.3	.48305
.4	185.4	.49940	.8	30.8	.49083	.2	7.82	18.7	.48284
.5	174.2	.49910	.9	29.4	.49057	.3	7.53	18.02	.48261
.6	164.8	.49884	18.0	28.1	.49032	.4	7.28	17.42	.48240
.7	155.2	.49859	.1	26.9	.49009	.5	7.03	16.82	.48217
.8	145.9	.49832	.2	25.7	1.48983	.6	6.76	16.18	.48195
.9	137.7	.49807	.3	24.7	.48959	.7	6.50	15.55	.48172
15.0	129.8	.49781	.4	23.6	.48935	.8	6.28	15.03	.48149
.1	122.2	.49756	.5	22.6	.48909	.9	6.10	14.60	.48127
.2	116.4	.49735	.6	21.7	.48885	22.0	5.89	14.09	.48103
.3	109.4	.49706	.7	20.7	.48860	.1	5.72	13.69	.48080
						.2	5.50	13.16	.48056
						.3	5.31	12.70	.48031

* Temperature correction factors for viscosity given in foregoing table.
† Temperature correction for refractive index is −0.00023 per degree.

shows the curve of log of water content plotted against viscosity as a nearly straight line, but the curves of log of water content against refractive index and log of viscosity against refractive index are somewhat more



Courtesy of Ind. Eng. Chem., Anal. Ed. 1939, **11**, 130

FIG. 142. Oppen and Schuette Modification of Gibson-Jacobs Viscometer.

curved, although not sufficiently to cause a considerable error.

Oppen and Schuette Viscometric Method.[63] APPARATUS. The *Viscometer* is simple in construction and easily operated. The illustration (Fig. 142) shows a calibrated Pyrex tube 25 mm. outside (21.2 mm. inside) diameter fitted with a tube, with an inside diameter of 3 mm., for introduction of steel balls. The metal mounting is not shown.

Constant Temperature Bath. The frame and tube are placed in a diffusely illuminated glass bath provided with a stirrer and surrounded by insulation in which an observation mirror is cut.

Steel Balls, 1.6 mm. in diameter.

PROCESS. Fill the 25-mm. tube to the highest calibration with the honey at 40° and adjust the 3-mm. tube so that the end reaches the mark 6 cm. from the top. Introduce the *steel ball* through the 3-mm. tube, allowing it to pass through the adjacent 5-cm. section to acquire velocity. If the honey is very viscous, note the time it takes to pass through the first and third of the next three 5-cm. sections. In this way duplicate readings are obtained with the same ball, the average of which multiplied by 3 gives the total time for the combined (15-cm.) sections. With less viscous honey, note the time required to pass through the whole 15-cm. measurement zone.

CALCULATION. Calculate the moisture content (W) by solving the following equation:

$$W = \frac{62{,}500 - 156.7T}{T(\log V_T + 1) - 2.287(313 - T)}$$

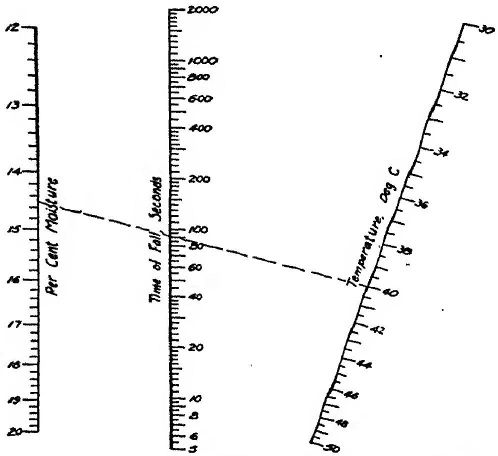
in which V_T is the viscosity (time of fall in seconds through 15 cm.) at the temperature T.

To facilitate calculation, Oppen and Schuette give a graph with degrees Centigrade as abscissas and time-of-fall in seconds as ordinates, showing percentages of moisture at the intersections.

A more convenient nomograph (Fig. 143), devised by Davis,[64] of Wayne University, Detroit, appears herewith. A straightedge, intersecting the time-of-fall and temperature as found, shows the percentage of moisture on the third scale.

EXAMPLES. In 29 samples varying from 12.31 to 19.73% of moisture and in viscosity at 40° from 15.8 to 310.3, the mean difference

between the viscometric and the A.O.A.C. evaporation method, found by Oppen and Schuette, ranged from −0.7 to +0.3%.



*Courtesy of Chem.-Anal. 1940, **29**, 87*

FIG. 143. Davis Nomograph.

POLARIZATION

Direct Polarization Method. Dissolve the normal weight (26 g. for instruments with the Ventzke scale) of the sample in water, clear with 5 to 10 ml. of *alumina cream* (use no lead subacetate solution), make up to 100 ml., filter, polarize at once at 20° in a 200-mm. tube, and record the reading as *immediate polarization.*

If immediate polarization is not desired, before making up to the mark neutralize with *ammonium hydroxide*, heat to boiling, and add a few drops of *ammonium hydroxide* to destroy mutarotation (multirotation, birotation) due to the high concentration. If this is done after taking the immediate reading, it is necessary to readjust the volume to 100 ml. at 20°. Addition of *solid sodium carbonate* to slight alkaline reaction after the immediate polarization, as recommended in the official method, destroys the mutarotation without changing the dilution. The polariscopic reading after destroying the mutarotation is the true or constant polarization.

Invert Polarization Method. Dilute 50 ml. with 25 ml. of water, add 10 ml. of *20% hydrochloric acid* (sp.gr. 1.1029), invert at 67° as described in Part II, E2, take the readings in a 200-mm. tube at 20 and 87°, and multiply the readings by 2.

CALCULATION of the sucrose content from the polarization data by formula is inaccurate because of the high invert sugar content, hence recourse must be taken to a reduction copper method (Part I, C6*a*). The calculation of the percentage of commercial glucose, as given below, from polarization data is also only approximate, but no more accurate method is available.

REDUCING SUGARS

Copper Reduction Methods. Determine reducing sugars by the Munson and Walker or Lane and Eynon methods (Part I, C6*a*, Reducing Sugars) in 25 ml. of a solution, prepared (1) by diluting to 250 ml. 10 ml. of the cleared solution used for the direct polarization, as above directed, or (2) by weighing a new portion of 25 g. into a 100-ml. volumetric flask, clearing with *alumina cream*, filtering, and diluting 10 ml. of the filtrate to 250 ml. The quantity of the sample represented by the 25 ml. in the former case is 0.26 g., in the latter case 0.25 g., which is a more convenient quantity for calculation than 0.26 g.

Express the result in terms of invert sugar.

LEVULOSE

Wiley Polarimetric Method.[65] Determine the direct polarization at 20° (constant), the invert polarization at 20° and 87°, and calculate the levulose by the following formula

$$r = \frac{100(1.0315P - P')}{26 \times 2.3919} = \frac{100(1.0315P - P')}{62.19}$$

in which L is per cent of levulose, P' and P are the direct readings at 20 and 87° respectively, 2.3919 is the variation between 20 and 87° in the polarization of 1 g. of levulose in 100 ml. of solution, and 1.0315 is the conversion factor for volume at 20° into volume at 87°.

Dextrose

Browne Calculation Method.[66] Calculate by the following formula:

$$D = R - 0.915L$$

in which D is per cent of dextrose, R is per cent of reducing sugar calculated as levulose, and L is per cent of levulose.

Sucrose

Browne Copper Reduction Method.[67] Neutralize with *sodium carbonate* 10 ml. of the solution obtained after inversion (see Polarization above). Dilute to 250 ml. in a volumetric flask and determine the per cent of invert sugar in 25 ml. (0.26 g. of the sample) by the Munson and Walker or Lane and Eynon methods (Part I, 6*a*, Reducing Sugars).

Subtract from the result the per cent of invert sugar determined before inversion as directed under Reducing Sugars above, multiply by 0.95 (which is molecular weight of sucrose ÷ molecular weight of invert sugar) to convert into per cent of sucrose.

A maximum limit of 8% of sucrose has been fixed in the U. S. Standards as a safeguard against adulteration with commercial sugar. Honey made by bees fed on sugar or working in the vicinity of sugar factories may contain 16% of sucrose or even more.

Dextrin

Dextrin occurs in extremely variable amount in honey. In one sample of Hawaiian honey Browne reported 10.01%.

Browne Alcohol Precipitate Gravimetric Method.[68] PROCESS. Tare a 100-ml. volumetric flask, weigh into it 8 g. of the sample (4 g. of the dark-colored honeydew honey), add 4 ml. of water, then add with continual shaking *absolute ethanol* sufficient to fill to the mark. Mix well and allow to stand for 24 hours or until the dextrin has deposited on the sides and bottom of the flask and the ethanol is perfectly clear. Decant onto a filter and wash the precipitate in the flask and on the filter with 10 ml. of cold *ethanol*. Pour boiling water into the flask, rotate to aid the solution of the dextrin, then pour the ethanolic solution on the filter, collecting the filtrate in a tared dish, repeating the treatment until all the dextrin in the form of solution is collected in the dish. Evaporate the ethanol and dry the residue to constant weight in a boiling water oven or, if the amount is considerable, in a vacuum oven at 70° under a pressure not exceeding 50 mm. of mercury. Dissolve the residue in water and make up to the mark in a volumetric flask of such capacity as to obtain an approximately 1% solution.

CALCULATION. Determine the weight of reducing sugar calculated as invert sugar by direct copper reduction, as directed above under Reducing Sugars, and of sucrose, from the data by direct and invert copper reduction as directed under Sucrose above.

Subtract the sum of the weights of the two sugars and ash from the weight of ethanol precipitate; the remainder is the dextrin.

Commercial Glucose

Beckmann Iodine Test.[69] In this test, advantage is taken of the presence in glucose of erythro- or amylodextrin as transition products between starch and dextrose, which is the ultimate product of the acid conversion. Iodine solution, which colors starch and soluble starch a deep blue, colors the intermediate products various combinations of blue and red.

Prepare a mixture of equal volumes of the

sample and water and add to the mixture a few drops of *potassium iodide-iodine solution*. The formation of a purple or red color shows the probable presence of commercial glucose, but a negative reaction does not necessarily prove the absence of glucose, since the high conversion product may not contain the intermediate products on which the test depends. In case of doubt, Beckmann recommends making the test on a solution of the alcohol precipitate (see Dextrin above).

None of the 100 samples of genuine honey examined by Browne [70] responded to the test.

Polariscopic Method. The polariscopic calculation method for the determination of commercial glucose (Part II, E4), which yields only approximate results in the analysis of molasses and related products, is still more inaccurate when applied to honey. The greater proportion of invert sugar and the presence of variable amounts of dextrin in both glucose and honey introduce complications.

Browne Formula Method.[71] Browne based his formula on the constant 26.7, which is the average difference between the invert reading after inversion at 20° and 87° and on 77 which is the average per cent of invert sugar in pure honey after inversion. He found that it gave better results than the European formulas based on -17.5 and $+17.5$ which are respectively the readings for pure honey at 20° and commercial glucose.

The formula for calculation of the per cent of pure honey (H) and the difference formula for calculating the per cent of commercial glucose (G) follow:

$$H = \frac{100(P' - P) \times 77}{26.7 \times I} = \frac{288.4(P' - P)}{I}$$

$$G = 100 - \frac{288.4(P' - P)}{I}$$

in which P' and P are the Ventzke readings of the inverted honey at 87° and 20° respectively and I is the per cent of invert sugar in the honey after inversion.

Artificial Invert

Since the greater part of natural honey consists of dextrose and levulose in equal molecular proportions, the detection of invert sugar made commercially by heating sugar sirup with dilute acid is practically impossible by polariscopic or copper reducing methods. Certain colorimetric tests, based on the presence of certain substances formed in small quantities during manufacture, follow.

Browne Aniline Acetate Test.[72] Reagent. *Aniline Acetate Reagent.* Mix by shaking 5 ml. of aniline and 5 ml. of water, then add 2 ml. of glacial acetic acid or a sufficient amount just to clear the emulsion. The solution must be freshly prepared.

Process. To 5 ml. of a mixture of equal volumes of honey and water contained in a test tube, add 1 to 2 ml. of *aniline acetate reagent* in such a manner that it flows down the sides of the test tube and forms a layer over the diluted honey.

A red ring formed, when the test tube is gently agitated, at the juncture of the two liquids and gradually extended to the whole lower solution is indicative of artificial invert sugar.

The reacting substance is furfural formed during the heating at high temperatures in the manufacturing process. Boiled honey may also form furfural, but since heating injures the flavor boiling is not practiced.

Feder Aniline Chloride Test.[73] Like the Browne test, this depends on the presence of furfural.

Reagent. *Aniline Chloride Solution.* Mix 100 ml. of aniline with 30 ml. of 25% HCl. The reagent must be freshly prepared.

Process. Stir 5 g. of honey in a porcelain dish with 2.5 ml. of *aniline chloride solution* until the mixture is homogenous.

Artificial invert sugar causes the immedi-

ate formation of a vivid red color. Feder states that the color fades after 15 minutes; in the Tentative A.O.A.C. Method it is stated that at first it is orange-red, then dark red.

Fiehe-Bryan Resorcinol Test.[74] Shake vigorously 10 ml. of a mixture of equal volumes of the sample and water in a test tube with 5 ml. of *ether* and allow to stand until the two liquids separate. Transfer 2 ml. of the ether solution to a small test tube, add a large drop of *1% resorcinol solution in hydrochloric acid*, and shake.

Artificial invert sugar solution is indicated by the formation of a cherry-red color, whereas a faint orange or rose color may be due to heating to dissolve the crystals that form in the honey on standing.

Fiehe-Nelson Resorcinol Test.[75] E. K. Nelson of the U. S. Bureau of Chemistry and Soils proceeds as follows. Dissolve 20 g. of honey in 20 ml. of water and extract the solution rapidly with *ether* in a Palkin-Watkins extractor for 30 minutes. Concentrate the ether to about 5 ml. and transfer to a test tube. Add 2 ml. of freshly prepared *1% resorcinal solution in hydrochloric acid* and shake immediately.

Note the color after 5 minutes. Pure honey gives an extremely faint pink; a mixture of pure honey with 2% invert sugar gives a dark red color.

Ley Ammoniacal Silver Nitrate Test.[76] Reagent. *Silver Reagent*. To a solution of 10 g. of $AgNO_3$ in 100 ml. of water, add 20 ml. of 15% NaOH solution. Collect the silver oxide precipitate on a filter, wash with 400 ml. of water, then dissolve in 10% NH_4OH, and make up with the NH_4OH to 115 g. Keep in a glass-stoppered bottle away from the light.

Process. To 5 ml. of a solution of 1 part of the sample and 2 parts of water in a test tube, add 5 drops of *silver reagent*, close with a plug of cotton, and heat immediately on a steam bath away from the direct sunlight. Observe the color after heating 5 minutes.

If the honey is genuine, the solution is dark colored, not directly transparent, and more or less fluorescent in reflected light; shaking develops a clear brownish red color on the surface of the liquid and a more or less characteristic brownish or yellowish green tint on the surface of the test tube. If the honey is not pure, the color of the liquid is dirty brown or black and the characteristic greenish tint does not develop on shaking.

Browne found that the brown color of the liquid, varying from light red to purplish, was quite characteristic—more so than the green after-tint. The test is not infallible, but has some value in conjunction with other tests.

Acids

Titrate 10 g. of the sample with *0.1 N sodium* or *potassium hydroxide solution*, using *phenolphthalein indicator*. Calculate as per cent of formic acid or express results in milliliters of the standard alkali per 100 g. of honey. Acids other than formic may be present in sound, normal honey. When it sours, acetic acid develops.

Ash

Ignite 10 g. of the sample below redness to a white ash, resorting to leaching, evaporation and ignition of the charcoal if necessary. See Part I, C2*f*, for further details and methods of determination of soluble and insoluble ash, also alkalinity.

7. CONFECTIONERY

The term confectionery is not mentioned in the U. S. standards. It is broader in its scope than candy which is defined as follows: "Candy is a product made from a saccharine substance or substances, with or without the addition of harmless coloring, flavoring, or

filling materials, and contains no terra alba, barytes, talc, chrome yellow, or other mineral substances, or poisonous colors or flavors, or other ingredients deleterious or detrimental to health, or any vinous, malt, or spirituous liquor or compound, or narcotic drug."

The term filling materials suggests a diluent, yet shredded cocoanut, nuts, and dried fruits, often present in candy, are neither saccharine nor filling materials. Starch in some candies may be classed as a filler, but in others often serves a useful purpose such as a non-hygroscopic vehicle for flavors.

Many forms of confectionery may also be termed candy. Other forms are intermediate between candy and pastry (e.g., macaroons) or are partly one and partly the other (e.g., Nabisco wafers).

Whatever the verdict of standards committees or lexicographers, both candy and confectionery are solid food delicacies characterized by their high content of one or more sugars. Other vegetable constituents may be nuts, fruits, cereals (e.g., puffed cereals), agar-agar, gum arabic, gum tragacanth, and starch paste. Among the animal food constituents are butter and other fats, dried milk, and gelatin.

Analysis of Confectionery. No set rules can be set down for the analysis of confectionery and candy since, like pastries and cake, the combinations are infinite. The selection of the method or combination of methods devolves on the analyst who must have a comprehensive knowledge of trade usage, properties of constituents, and analytical methods for individual constituents or groups of constituents. The following paragraphs are suggestive, not didactic.

Mechanical Separation

When parts of different composition are in layers (e.g., sugared or chocolate almonds), these parts may be carefully separated without wetting, weighed, and analyzed.

Solubility Separation

When nut meats or dried fruits are embedded in a saccharine material, solution of the latter and washing and drying of the insoluble constituents are essential, using warm but not hot water since cold water reduces the time for absorption of sugars but increases the penetration. Approximate correction may be made for the loss of soluble constituents of nuts and dried fruits as determined by separate experiment. Determination of moisture on the original sample and the separated nuts and fruits and of solids in the insoluble matter supplies data for further adjustment of the analysis.

Greater difficulties are encountered in the presence of finely ground, more or less insoluble materials, such as chocolate or peanut butter or constituents like milk powder or butter which cause a turbidity that slows filtration or gives a turbid filtrate. A turbid filtrate does not interfere with sugar determinations by the usual methods and the loss of proteins may be ignored if separate determination of protein is made on the original material. Little if any fat will pass through a wet paper, but if there is doubt the filtrate may be shaken with ether or petroleum ether.

The insoluble residue may be dried and weighed on a tared paper and fat determination made thereafter by extraction with ether in a Johnson extractor, as a check on the results by direct extraction.

Protein

If nut meats, milk powder, or other nitrogenous materials are present, determine the nitrogen and calculate the protein. Use the factor 6.38, if the protein is entirely from milk powder.

Dissolve a weighed amount of the material in water and determine fat by the Röse-Gottleib method (Part II, G1).

Reducing Sugars

Determine by copper reduction (Part I, C6*a*) in a deleaded aliquot of the solution prepared for polarization.

Sucrose

Weigh a normal amount of the sample, defecate if necessary with *lead subacetate* and *alumina cream*, and polarize directly at 20° and after inversion at 20° and 87°. Calculate by Clerget's formula or a modification (Part II, E2, above).

Commercial Glucose

Calculate from the polarization at 87° by the formula given under Molasses and Sirup above.

Lactose

If milk solids, but not chocolate or cocoa, are present, use the method for Sweetened Condensed Milk (Part II, G3). If milk solids and chocolate or cocoa are present, use the method given in Part II, I3.

Colors

In no class of foods is coloring so common and so excusable. Methods for detection are given in Part I, C12.

Flavors

Imitation fruit flavors are usually detected by their organoleptic characters; identification by chemical analysis requires a large amount of material and special experience. Still more difficult is the distinction of synthetic vanillin or coumarin from the natural, because of the small amount and interfering substances.

Mineral Constituents

See Part I, C8*a* and *b*.

There is no evidence that calcium sulfate or other mineral adulterant is now added to confectionery. If such were present, it would be evident by its slight solubility. The detection of added alkali carbonate in Dutch process cocoa is considered in Part II, I3.

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F. ALCOHOLIC BEVERAGES

Liebig regarded fermentation as a purely chemical process and ignored the biological theories of Pasteur and others which have since been accepted. Kröber, by his classical researches, has more recently shown that the ferments of yeast may act without the intervention of the growth of the cells, thus returning in a sense to the purely chemical theories of Liebig.

The carbon dioxide formed during fermentation is either allowed to escape or, as in the case of malt liquors and effervescent wines, is confined, at least in part, by tight casks or corked bottles.

Natural wines cannot contain more than about 18% of ethanol, as the yeast plant ceases to grow after that strength has been reached. By adding ethanol, fortified wines, such as sherry and port, are obtained and by distillation any desired alcoholic strength can be secured. Cognac or French brandy is the distillate from wine; cider brandy, from fermented cider; whiskey and gin, from fermented grain infusions; and rum, from diluted and fermented molasses.

Theoretically over 51% of invert sugar is obtainable as ethanol, but practically under the most favorable conditions the yield is less than 49%, the remainder going to form glycerol, succinic acid, and various higher alcohols which make up the fusel oil of distilled liquors.

1. WINE AND CIDER

Wine has been made—and adulterated—since prehistoric times. A vast amount of study has been devoted to the analysis of wines by European chemists, partly to distinguish the genuine from the false and partly in connection with the control of manufacturing processes and the detection of spoilage.

Fermentation. Grape must, cider, and other fruit juices ferment through the action on the invert sugar of the enzyme *zymase* of the wild yeast plants *Saccharomyces ellipsoideus, S. apiculatus*, etc., which naturally occur on the outside of the fruit and find their way into the expressed fruit juices, the reaction being as follows:

$$+ 2CO_2 \uparrow$$

Chemical Composition. *Grape Must and Apple Juice.* Before fermentation the fruit juice (must) consists essentially of a liquid sweetened with sucrose and invert sugar in varying proportion and acidified with organic acids (tartaric and malic or their acid salts in must, malic, and other acids in apple juice) together with minor but exceedingly valuable constituents, such as tannins, flavors (methyl anthranilate in American grapes; various esters, acids, aldehydes, and alcohols in must and apple juice), colors, and mineral matter. Preservatives may be present.

Wine and Cider. During fermentation the sugars largely pass into ethanol and various substances, such as glycerol and lactic acid which appear as by-products. Spoilage is accompanied by the formation of acids, notably acetic which increases the amount of volatile acid from traces to significant amounts.

	Ethanol	Extract	Total Acidity as Tartaric	Volatile Acids as Acetic	Sugar	Glycerol	Ash	Phosphoric Acid
Claret	8.16	2.42	0.58	0.10	0.23	0.73	0.25	0.029
Rhine wine	8.12	2.91	0.77	0.05	0.23	0.85	0.20	0.045
Sauterne	9.48	3.03	0.66	0.09	0.84	0.97	0.25	0.032
Sherry	16.09	4.06	0.41	....	2.40	0.51	0.46	0.028
Champagne (dry)	10.42	2.36	0.61	....	0.53	0.71	0.14	

The average composition of wines in terms of grams per 100 ml., compiled from analyses by König,[1] are given in the table above.

In addition to the constituents given in the table, the following may be determined: potassium sulfate (used in sulfured wines), sodium chloride, nitrates, tannin, preservatives, and colors.

Sample

The deposit of argols, or crude cream of tartar, formed during fermentation, by reason of their insolubility in the ethanol largely remain in the tanks or casks on racking off the wine. Separate any deposit that forms in the bottle by decanting and filtering. Determine its percentage, if desired, by collecting on a weighed paper and drying. Effervescent wines and ciders are treated like beer in the determination of carbon dioxide and the preparation of decarbonated samples for the remainder of the analysis.

Specific Gravity

Determine at 20° in a pycnometer or with a hydrometer, both accurately calibrated at 20°, as directed for decarbonated malt liquors below.

Extract

(Solids)

The following methods have been adopted

Density Method. Obtain the specific gravity of the "dealcoholized sample" (D) by the following formula:

$$D = S + 1 - A$$

in which S is the specific gravity of the sample and A is the specific gravity of the distillate obtained in the determination of ethanol. Find the per cent by weight of the extract in the "dealcoholized sample" corresponding to D in the Plato table and multiply by S; the product is the grams of extract per 100 ml. (g. per 100 ml.) in the sample.

The per cent by weight of extract corresponding to D may also be found in one of the columns headed Degrees Brix or Sucrose Per Cent of the Domke table, Part II, E3, which replaces the Plato table in this work.

Evaporation Method. Employ 50 g. of dry wines or 25 g. of sweet wines with 3 to 6 g. per 100 ml. of extract. Evaporate in a flat-bottom dish 85 mm. in diameter on a water bath to a sirup and dry 2 to 5 hours in a boiling water oven. Cool in a desiccator and weigh. The procedure is essentially that of the German methods, except that the limit is 6 (not 4) g. per 100 ml. and only 25 ml. of the sample are employed.

If more than 6 g. per 100 ml. of extract is present, follow the density method to avoid the error due to the decomposition of levulose and slow evaporation of glycerol.

Notes. The complicated details of certain

methods are designed to avoid the error attributed to decomposition of levulose, but also due in part, if not largely, to other causes. von der Heide and Schwenke[3] note that in the evaporation lactic acid disappears in part, tartaric acid passes into the meta-form, and malic acid into malonic acid, but succinic acid remains unaltered; also that volatile acids combine with glycerol to form esters. They consider the loss of glycerol as inconsiderable, but other authors found a loss up to 35%.

Crampton[4] found that 25 samples, after the initial drying, sustained an additional average loss of 0.60% on drying to constant weight.

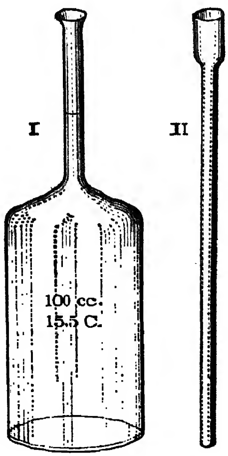


FIG. 144 I Pycnometer; II Delivery Tube.

ETHANOL

(Alcohol)

Distillation-Density Method. The procedure is the same for all kinds of alcoholic liquors, except that (1) the addition of 0.1 to 0.12 g. of calcium carbonate or standard alkali to neutral reaction is necessary if the wine or cider has partly turned into vinegar, (2) effervescent liquors need decarbonating, and (3) only 25 g. or ml. of distilled liquors and cordials are employed.

APPARATUS. The *Distillation Apparatus* used for determining the volatile fatty acids (Fig. 108) is suitable except that a delicate 100-ml. pycnometer (Fig. 144, *I*) is substituted for the wide-mouth receiving flask and the condenser tube is connected at the lower end by means of a rubber tubing with a delivery tube (Fig. 144, *II*), the lower part of which is of such a size that it readily passes through the neck of the pycnometer. The height of the pycnometer should be such that it can stand erect on the balance pan and the inside of the neck should be 5 mm. It is calibrated to contain 100 g. of water at 20°. Fig. 145 shows the complete apparatus set up ready for use.

PROCESS. If the liquor is effervescent, pour from one glass to another until no more bubbles of carbon dioxide escape; if froth persists, remove by filtering. Weigh the clean dry pycnometer and fill with the sample so that the lower meniscus is exactly at the mark; use the delivery tube as a funnel. Weigh the pycnometer plus sample, then transfer the sample to a 300-ml. flask, rinsing with 50 ml. of water. If the sample contains an abnormal amount of acetic acid, neutralize with *1.0 N sodium hydroxide solution*. Only when necessary to prevent frothing over during distillation, add a very little *tannin*. Connect the flask with the condenser by means of the bulb tube.

Introduce into the pycnometer the delivery tube, attach the latter to the condenser tube, and turn on the water. Heat cautiously to boiling and continue to boil until the pycnometer is filled nearly to the bottom of the neck. Detach the delivery tube, rinse with a few drops of water, and add water nearly to the graduation mark. Mix by shaking and place in a bath of water at 20°, taking care that the water covers the pycnometer to the height of the liquid within. After allowing to stand in the bath at least 15

minutes, remove the pycnometer, without delay add water at 20° by means of a small pipet until the lower meniscus is exactly at the mark, dry off the outside surface, and weigh.

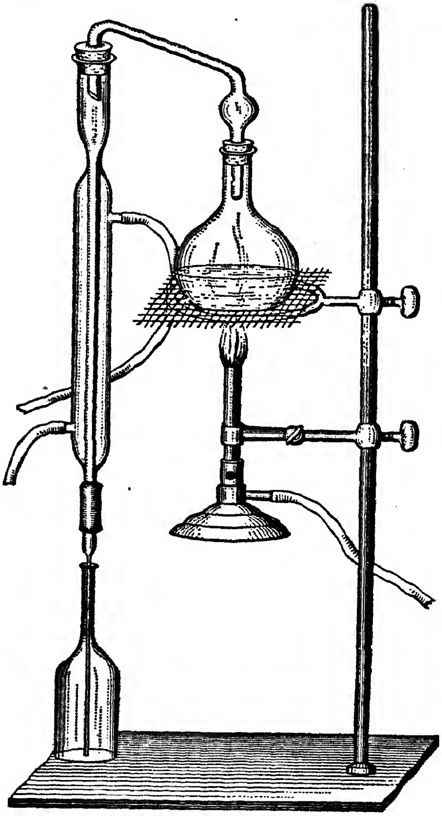


FIG. 145. Ethanol Distillation Apparatus.

CALCULATION. Subtract from the total weight the weight of the empty pycnometer, thus obtaining the weight of the distillate, which divided by 100 gives the apparent specific gravity. In the first table herewith find the per cent of ethanol by volume corresponding to the apparent specific gravity, also in the second table find the percentage of ethanol by weight corresponding to the per cent by volume. As the volume of both the sample and the distillate is 100 ml., the percentage by volume in both is the same. To obtain the percentage by weight in the sample, multiply the weight of the distillate by the percentage of ethanol by weight contained in it and divide by the weight of the sample.

If no anti-foam or neutralizer has been added, reserve the residue in the distillation flask for the determination of extract (solids) as directed above.

Distillation-Refraction Method. Proceed with the distillation as in the distillation-specific gravity method. Make up the distillate in the pycnometer to 100 ml. at 17.5° or 20° C. Take the reading on the scale of the Zeiss immersion refractometer, find the corresponding per cent of ethanol by volume in the third table herewith, and the corresponding per cent of ethanol by weight in the second table herewith. Calculate the per cent of ethanol by volume and weight as directed for the distillation-specific gravity method.

Readings of scales other than that of the Zeiss instrument must be converted into refractive indices and the corresponding per cents of ethanol obtained from the tables.

Sémichon and Flanzy Dichromate Volumetric Method.[5] The method, as developed at the Paris Municipal Laboratory, consists essentially of oxidation to acetic acid by standard potassium dichromate and titration of the excess with ferrous ammonium sulfate.

REAGENTS. *Standard Potassium Dichromate Solution.* Dissolve 33.832 g. of $K_2Cr_2O_7$ in water and dilute to 1 liter at 15°; **1** ml. = 7.943 mg. of ethanol or 1% by volume.

Standard Mohr's Salt Solution. Dissolve 135.310 g. of crystalline $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 20 ml. of H_2SO_4 (sp.gr. 1.8354) in water and dilute to 1 liter; 2 ml. = **1** ml. of the standard

ETHANOL BY VOLUME AT 15.56° C. FROM APPARENT SPECIFIC GRAVITY AT 20° C. (N.B.S.) *

Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent
1.0000	0.00	0.9930	4.84	0.9860	10.36	0.9790	16.46	0.9720	22.96	0.9650	29.03
0.9999	.07	.9929	.91	.9859	.44	.9789	.55	.9719	23.05	.9649	.11
.9998	.13	.9928	.98	.9858	.53	.9788	.64	.9718	.15	.9648	.19
.9997	.20	.9927	5.06	.9857	.61	.9787	.73	.9717	.24	.9647	.27
.9996	.26	.9926	.13	.9856	.69	.9786	.83	.9716	.33	.9646	.35
.9995	.33	.9925	.21	.9855	.78	.9785	.92	.9715	.42	.9645	.44
.9994	.40	.9924	.28	.9854	.86	.9784	17.01	.9714	.51	.9644	.52
.9993	.46	.9923	.36	.9853	.94	.9783	.10	.9713	.60	.9643	.60
.9992	.53	.9922	.43	.9852	11.03	.9782	.20	.9712	.69	.9642	.68
.9991	.60	.9921	.51	.9851	.11	.9781	.29	.9711	.78	.9641	.76
.9990	.66	.9920	.58	.9850	.19	.9780	.38	.9710	.87	.9640	.85
.9989	.73	.9919	.66	.9849	.28	.9779	.47	.9709	.95	.9639	.93
.9988	.80	.9918	.73	.9848	.36	.9778	.57	.9708	24.04	.9638	30.01
.9987	.87	.9917	.81	.9847	.45	.9777	.66	.9707	.13	.9637	.09
.9986	.93	.9916	.88	.9846	.53	.9776	.75	.9706	.22	.9636	.17
.9985	1.00	.9915	.96	.9845	.61	.9775	.84	.9705	.31	.9635	.25
.9984	.07	.9914	6.03	.9844	.70	.9774	.94	.9704	.40	.9634	.34
.9983	.14	.9913	.11	.9843	.78	.9773	18.03	.9703	.49	.9633	.42
.9982	.20	.9912	.18	.9842	.87	.9772	.12	.9702	.58	.9632	.50
.9981	.27	.9911	.26	.9841	.95	.9771	.22	.9701	.66	.9631	.58
.9980	.34	.9910	.34	.9840	12.04	.9770	.31	.9700	.75	.9630	.66
.9979	.41	.9909	.41	.9839	.12	.9769	.41	.9699	.84	.9629	.74
.9978	.48	.9908	.49	.9838	.21	.9768	.50	.9698	.93	.9628	.82
.9977	.54	.9907	.57	.9837	.29	.9767	.59	.9697	25.01	.9627	.89
.9976	.61	.9906	.65	.9836	.38	.9766	.69	.9696	.10	.9626	.97
.9975	.68	.9905	.73	.9835	.47	.9765	.78	.9695	.19	.9625	31.05
.9974	.75	.9904	.80	.9834	.55	.9764	.88	.9694	.28	.9624	.13
.9973	.81	.9903	.88	.9833	.64	.9763	.97	.9693	.36	.9623	.20
.9972	.88	.9902	.96	.9832	.73	.9762	19.07	.9692	.45	.9622	.28
.9971	.95	.9901	7.04	.9831	.81	.9761	.16	.9691	.54	.9621	.36
.9970	2.02	.9900	.12	.9830	.90	.9760	.26	.9690	.62	.9620	.44
.9969	.09	.9899	.19	.9829	.99	.9759	.35	.9689	.71	.9619	.52
.9968	.15	.9898	.27	.9828	13.07	.9758	.45	.9688	.80	.9618	.59
.9967	.22	.9897	.35	.9827	.16	.9757	.54	.9687	.89	.9617	.67
.9966	.29	.9896	.43	.9826	.25	.9756	.64	.9686	.98	.9616	.75
.9965	.37	.9895	.51	.9825	.34	.9755	.73	.9685	26.06	.9615	.82
.9964	.43	.9894	.59	.9824	.43	.9754	.83	.9684	.15	.9614	.90
.9963	.50	.9893	.67	.9823	.51	.9753	.92	.9683	.24	.9613	.98
.9962	.57	.9892	.75	.9822	.60	.9752	20.02	.9682	.33	.9612	32.05
.9961	.64	.9891	.82	.9821	.68	.9751	.11	.9681	.41	.9611	.13
.9960	.70	.9890	.90	.9820	.77	.9750	.20	.9680	.50	.9610	.21
.9959	.77	.9889	.98	.9819	.86	.9749	.30	.9679	.59	.9609	.28
.9958	.84	.9888	8.06	.9818	.95	.9748	.39	.9678	.67	.9608	.36
.9957	.91	.9887	.15	.9817	14.04	.9747	.48	.9677	.76	.9607	.43
.9956	.98	.9886	.23	.9816	.13	.9746	.58	.9676	.84	.9606	.51
.9955	3.05	.9885	.31	.9815	.22	.9745	.67	.9675	.93	.9605	.58
.9954	.12	.9884	.39	.9814	.30	.9744	.76	.9674	27.01	.9604	.66
.9953	.19	.9883	.47	.9813	.39	.9743	.86	.9673	.10	.9603	.73
.9952	.26	.9882	.55	.9812	.48	.9742	.95	.9672	.19	.9602	.81
.9951	.33	.9881	.63	.9811	.57	.9741	21.04	.9671	.27	.9601	.88
.9950	.40	.9880	.71	.9810	.66	.9740	.14	.9670	.36	.9600	.96
.9949	.47	.9879	.79	.9809	.75	.9739	.23	.9669	.44	.9599	33.03
.9948	.54	.9878	.88	.9808	.84	.9738	.32	.9668	.52	.9598	.10
.9947	.61	.9877	.96	.9807	.93	.9737	.41	.9667	.61	.9597	.18
.9946	.68	.9876	9.04	.9806	15.02	.9736	.50	.9666	.69	.9596	.25
.9945	.76	.9875	.13	.9805	.11	.9735	.60	.9665	.77	.9595	.32
.9944	.83	.9874	.21	.9804	.20	.9734	.69	.9664	.86	.9594	.40
.9943	.90	.9873	.29	.9803	.28	.9733	.78	.9663	.94	.9593	.47
.9942	.97	.9872	.38	.9802	.37	.9732	.87	.9662	28.02	.9592	.54
.9941	4.04	.9871	.46	.9801	.46	.9731	.96	.9661	.11	.9591	.62
.9940	.11	.9870	.54	.9800	.55	.9730	22.05	.9660	.19	.9590	.69
.9939	.18	.9869	.62	.9799	.64	.9729	.14	.9659	.28	.9589	.76
.9938	.26	.9868	.70	.9798	.73	.9728	.24	.9658	.36	.9588	.84
.9937	.33	.9867	.79	.9797	.82	.9727	.33	.9657	.44	.9587	.91
.9936	.40	.9866	.87	.9796	.91	.9726	.42	.9656	.53	.9586	.98
.9935	.48	.9865	.95	.9795	16.00	.9725	.51	.9655	.61	.9585	34.05
.9934	.55	.9864	10.03	.9794	.10	.9724	.60	.9654	.69	.9584	.12
.9933	.62	.9863	.11	.9793	.19	.9723	.69	.9653	.78	.9583	.20
.9932	.69	.9862	.20	.9792	.28	.9722	.78	.9652	.86	.9582	.27
.9931	.77	.9861	.28	.9791	.37	.9721	.87	.9651	.94	.9581	.34

* *Bull. Natl. Bur. Standards* Vol. 9, No. 3, Reprint No. 197 via *Methods of Analysis*, A.O.A.C.

ETHANOL BY VOLUME AT 15.56° C. FROM APPARENT SPECIFIC GRAVITY AT 20° C. (N.B.S.)—*Continued*

Sp.Gr.	Per Cent	Sp. Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent
0.9580	34.41	0.9510	39.10	0.9440	43.33	0.9370	47.20	0.9300	50.81	0.9230	54.24
.9579	.48	.9509	.16	.9439	.39	.9369	.26	.9299	.86	.9229	.29
.9578	.56	.9508	.23	.9438	.44	.9368	.31	.9298	.91	.9228	.33
.9577	.63	.9507	.29	.9437	.50	.9367	.36	.9297	.96	.9227	.38
.9576	.70	.9506	.35	.9436	.56	.9366	.42	.9296	51.01	.9226	.43
.9575	.77	.9505	.41	.9435	.62	.9365	.47	.9295	.06	.9225	.48
.9574	.84	.9504	.48	.9434	.67	.9364	.52	.9294	.11	.9224	.53
.9573	.91	.9503	.54	.9433	.73	.9363	.58	.9293	.16	.9223	.57
.9572	.98	.9502	.60	.9432	.78	.9362	.63	.9292	.21	.9222	.62
.9571	35.05	.9501	.67	.9431	.85	.9361	.68	.9291	.26	.9221	.67
.9570	.12	.9500	.73	.9430	.90	.9360	.73	.9290	.31	.9220	.72
.9569	.19	.9499	.79	.9429	.96	.9359	.79	.9289	.36	.9219	.77
.9568	.26	.9498	.85	.9428	44.02	.9358	.84	.9288	.41	.9218	.81
.9567	.33	.9497	.91	.9427	.07	.9357	.89	.9287	.46	.9217	.86
.9566	.40	.9496	.98	.9426	.13	.9356	.94	.9286	.50	.9216	.91
.9565	.47	.9495	40.04	.9425	.18	.9355	48.00	.9285	.55	.9215	.96
.9564	.54	.9494	.10	.9424	.24	.9354	.05	.9284	.60	.9214	55.00
.9563	.61	.9493	.16	.9423	.30	.9353	.10	.9283	.65	.9213	.05
.9562	.68	.9492	.22	.9422	.35	.9352	.15	.9282	.70	.9212	.10
.9561	.75	.9491	.29	.9421	.41	.9351	.21	.9281	.75	.9211	.15
.9560	.82	.9490	.35	.9420	.46	.9350	.26	.9280	.80	.9210	.19
.9559	.88	.9489	.41	.9419	.52	.9349	.31	.9279	.85	.9209	.24
.9558	.95	.9488	.47	.9418	.58	.9348	.36	.9278	.90	.9208	.29
.9557	36.02	.9487	.53	.9417	.63	.9347	.41	.9277	.95	.9207	.34
.9556	.09	.9486	.59	.9416	.69	.9346	.47	.9276	52.00	.9206	.38
.9555	.15	.9485	.65	.9415	.74	.9345	.52	.9275	.05	.9205	.43
.9554	.22	.9484	.71	.9414	.80	.9344	.57	.9274	.10	.9204	.48
.9553	.29	.9483	.78	.9413	.86	.9343	.62	.9273	.15	.9203	.53
.9552	.36	.9482	.84	.9412	.91	.9342	.68	.9272	.20	.9202	.57
.9551	.42	.9481	.90	.9411	.97	.9341	.73	.9271	.25	.9201	.62
.9550	.49	.9480	.96	.9410	45.03	.9340	.78	.9270	.29	.9200	.67
.9549	.56	.9479	41.02	.9409	.08	.9339	.83	.9269	.34	.9199	.71
.9548	.63	.9478	.08	.9408	.14	.9338	.88	.9268	.39	.9198	.76
.9547	.69	.9477	.14	.9407	.19	.9337	.94	.9267	.44	.9197	.81
.9546	.76	.9476	.20	.9406	.25	.9336	.99	.9266	.49	.9196	.86
.9545	.83	.9475	.26	.9405	.30	.9335	49.04	.9265	.54	.9195	.90
.9544	.89	.9474	.32	.9404	.36	.9334	.09	.9264	.59	.9194	.95
.9543	.96	.9473	.38	.9403	.42	.9333	.14	.9263	.64	.9193	56.00
.9542	37.03	.9472	.44	.9402	.47	.9332	.19	.9262	.69	.9192	.04
.9541	.09	.9471	.50	.9401	.53	.9331	.25	.9261	.74	.9191	.09
.9540	.16	.9470	.56	.9400	.58	.9330	.30	.9260	.79	.9190	.14
.9539	.23	.9469	.62	.9399	.64	.9329	.35	.9259	.84	.9189	.18
.9538	.29	.9468	.68	.9398	.69	.9328	.40	.9258	.89	.9188	.23
.9537	.36	.9467	.74	.9397	.74	.9327	.45	.9257	.93	.9187	.28
.9536	.42	.9466	.80	.9396	.80	.9326	.50	.9256	.98	.9186	.32
.9535	.49	.9465	.86	.9395	.85	.9325	.55	.9255	53.03	.9185	.37
.9534	.56	.9464	.92	.9394	.91	.9324	.60	.9254	.08	.9184	.42
.9533	.62	.9463	.98	.9393	.96	.9323	.65	.9253	.13	.9183	.46
.9532	.69	.9462	42.04	.9392	46.01	.9322	.70	.9252	.18	.9182	.51
.9531	.75	.9461	.09	.9391	.07	.9321	.75	.9251	.22	.9181	.56
.9530	.82	.9460	.15	.9390	.12	.9320	.80	.9250	.27	.9180	.60
.9529	.88	.9459	.21	.9389	.18	.9319	.85	.9249	.32	.9179	.65
.9528	.95	.9458	.27	.9388	.23	.9318	.90	.9248	.37	.9178	.70
.9527	38.01	.9457	.33	.9387	.29	.9317	.95	.9247	.42	.9177	.74
.9526	.07	.9456	.39	.9386	.34	.9316	50.00	.9246	.47	.9176	.79
.9525	.14	.9455	.45	.9385	.39	.9315	.05	.9245	.52	.9175	.84
.9524	.20	.9454	.51	.9384	.45	.9314	.10	.9244	.56	.9174	.88
.9523	.27	.9453	.57	.9383	.50	.9313	.16	.9243	.61	.9173	.93
.9522	.33	.9452	.63	.9382	.56	.9312	.21	.9242	.66	.9172	.97
.9521	.39	.9451	.69	.9381	.61	.9311	.26	.9241	.71	.9171	57.02
.9520	.46	.9450	.74	.9380	.67	.9310	.31	.9240	.76	.9170	.07
.9519	.52	.9449	.80	.9379	.72	.9309	.36	.9239	.81	.9169	.11
.9518	.59	.9448	.86	.9378	.77	.9308	.41	.9238	.85	.9168	.16
.9517	.65	.9447	.92	.9377	.83	.9307	.46	.9237	.90	.9167	.21
.9516	.72	.9446	.98	.9376	.88	.9306	.51	.9236	.95	.9166	.25
.9515	.78	.9445	43.04	.9375	.94	.9305	.56	.9235	54.00	.9165	.30
.9514	.84	.9444	.09	.9374	.99	.9304	.61	.9234	.05	.9164	.35
.9513	.91	.9443	.15	.9373	47.04	.9303	.66	.9233	.09	.9163	.39
.9512	.97	.9442	.21	.9372	.10	.9302	.71	.9232	.14	.9162	.44
.9511	39.04	.9441	.27	.9371	.15	.9301	.76	.9231	.19	.9161	.48

Ethanol by Volume at 15.56° C. from Apparent Specific Gravity

Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent
0.9160	57.53	0.9090	60.68	0.9020	63.73	0.8950	66.69	0.8880	69.56	0.8810	72.34
.9159	.58	.9089	.72	.9019	.77	.8949	.73	.8879	.60	.8809	.38
.9158	.62	.9088	.77	.9018	.82	.8948	.77	.8878	.64	.8808	.42
.9157	.67	.9087	.81	.9017	.86	.8947	.81	.8877	.68	.8807	.46
.9156	.71	.9086	.86	.9016	.90	.8946	.85	.8876	.72	.8806	.50
.9155	.76	.9085	.90	.9015	.94	.8945	.90	.8875	.76	.8805	.53
.9154	.81	.9084	.94	.9014	.99	.8944	.94	.8874	.80	.8804	.57
.9153	.85	.9083	.99	.9013	64.03	.8943	.98	.8873	.84	.8803	.61
.9152	.90	.9082	61.03	.9012	.07	.8942	67.02	.8872	.88	.8802	.65
.9151	.94	.9081	.08	.9011	.11	.8941	.06	.8871	.92	.8801	.69
.9150	.99	.9080	.12	.9010	.16	.8940	.10	.8870	.96	.8800	.73
.9149	58.03	.9079	.17	.9009	.20	.8939	.15	.8869	70.00	.8799	.77
.9148	.08	.9078	.21	.9008	.24	.8938	.19	.8868	.04	.8798	.81
.9147	.13	.9077	.25	.9007	.28	.8937	.23	.8867	.08	.8797	.85
.9146	.17	.9076	.30	.9006	.33	.8936	.27	.8866	.12	.8796	.88
.9145	.22	.9075	.34	.9005	.37	.8935	.31	.8865	.16	.8795	.92
.9144	.26	.9074	.39	.9004	.41	.8934	.35	.8864	.20	.8794	.96
.9143	.31	.9073	.43	.9003	.45	.8933	.39	.8863	.24	.8793	73.00
.9142	.35	.9072	.47	.9002	.50	.8932	.43	.8862	.28	.8792	.04
.9141	.40	.9071	.52	.9001	.54	.8931	.47	.8861	.32	.8791	.08
.9140	.44	.9070	.56	.9000	.58	.8930	.52	.8860	.36	.8790	.12
.9139	.49	.9069	.60	.8999	.62	.8929	.56	.8859	.40	.8789	.16
.9138	.53	.9068	.65	.8998	.67	.8928	.60	.8858	.44	.8788	.19
.9137	.58	.9067	.69	.8997	.71	.8927	.64	.8857	.48	.8787	.23
.9136	.62	.9066	.74	.8996	.75	.8926	.68	.8856	.52	.8786	.27
.9135	.67	.9065	.78	.8995	.79	.8925	.72	.8855	.56	.8785	.31
.9134	.71	.9064	.82	.8994	.84	.8924	.76	.8854	.60	.8784	.35
.9133	.76	.9063	.87	.8993	.88	.8923	.80	.8853	.64	.8783	.39
.9132	.80	.9062	.91	.8992	.92	.8922	.84	.8852	.68	.8782	.43
.9131	.85	.9061	.96	.8991	.96	.8921	.89	.8851	.72	.8781	.47
.9130	.89	.9060	62.00	.8990	65.01	.8920	.93	.8850	.76	.8780	.50
.9129	.94	.9059	.04	.8989	.05	.8919	.97	.8849	.80	.8779	.54
.9128	.98	.9058	.09	.8988	.09	.8918	68.01	.8848	.84	.8778	.58
.9127	59.03	.9057	.13	.8987	.13	.8917	.05	.8847	.88	.8777	.62
.9126	.07	.9056	.17	.8986	.18	.8916	.09	.8846	.92	.8776	.66
.9125	.12	.9055	.22	.8985	.22	.8915	.13	.8845	.96	.8775	.70
.9124	.16	.9054	.26	.8984	.26	.8914	.17	.8844	71.00	.8774	.74
.9123	.21	.9053	.30	.8983	.30	.8913	.21	.8843	.04	.8773	.78
.9122	.25	.9052	.35	.8982	.35	.8912	.26	.8842	.08	.8772	.81
.9121	.30	.9051	.39	.8981	.39	.8911	.30	.8841	.12	.8771	.85
.9120	.34	.9050	.43	.8980	.43	.8910	.34	.8840	.16	.8770	.89
.9119	.39	.9049	.48	.8979	.47	.8909	.38	.8839	.20	.8769	.93
.9118	.43	.9048	.52	.8978	.51	.8908	.42	.8838	.24	.8768	.97
.9117	.48	.9047	.56	.8977	.56	.8907	.46	.8837	.27	.8767	74.01
.9116	.52	.9046	.60	.8976	.60	.8906	.50	.8836	.31	.8766	.05
.9115	.57	.9045	.65	.8975	.64	.8905	.54	.8835	.35	.8765	.08
.9114	.61	.9044	.69	.8974	.68	.8904	.58	.8834	.39	.8764	.12
.9113	.66	.9043	.73	.8973	.72	.8903	.62	.8833	.43	.8763	.16
.9112	.70	.9042	.78	.8972	.77	.8902	.67	.8832	.47	.8762	.20
.9111	.75	.9041	.82	.8971	.81	.8901	.71	.8831	.51	.8761	.24
.9110	.79	.9040	.86	.8970	.85	.8900	.75	.8830	.55	.8760	.28
.9109	.84	.9039	.91	.8969	.89	.8899	.79	.8829	.59	.8759	.32
.9108	.88	.9038	.95	.8968	.94	.8898	.83	.8828	.63	.8758	.35
.9107	.92	.9037	.99	.8967	.98	.8897	.87	.8827	.67	.8757	.39
.9106	.97	.9036	63.04	.8966	66.02	.8896	.91	.8826	.71	.8756	.43
.9105	60.01	.9035	.08	.8965	.06	.8895	.95	.8825	.75	.8755	.47
.9104	.06	.9034	.12	.8964	.10	.8894	.99	.8824	.79	.8754	.51
.9103	.10	.9033	.17	.8963	.15	.8893	69.03	.8823	.83	.8753	.55
.9102	.15	.9032	.21	.8962	.19	.8892	.07	.8822	.87	.8752	.58
.9101	.19	.9031	.25	.8961	.23	.8891	.11	.8821	.91	.8751	.62
.9100	.24	.9030	.30	.8960	.27	.8890	.15	.8820	.95	.8750	.66
.9099	.28	.9029	.34	.8959	.31	.8889	.19	.8819	.99	.8749	.70
.9098	.33	.9028	.38	.8958	.36	.8888	.23	.8818	72.03	.8748	.74
.9097	.37	.9027	.43	.8957	.40	.8887	.27	.8817	.07	.8747	.77
.9096	.41	.9026	.47	.8956	.44	.8886	.32	.8816	.10	.8746	.81
.9095	.46	.9025	.51	.8955	.48	.8885	.36	.8815	.14	.8745	.85
.9094	.50	.9024	.56	.8954	.52	.8884	.40	.8814	.18	.8744	.89
.9093	.55	.9023	.60	.8953	.56	.8883	.44	.8813	.22	.8743	.93
.9092	.59	.9022	.64	.8952	.60	.8882	.48	.8812	.26	.8742	.97
.9091	.64	.9021	.69	.8951	.65	.8881	.52	.8811	.30	.8741	75.00

ETHANOL BY VOLUME AT 15.56° C. FROM APPARENT SPECIFIC GRAVITY AT 20° C. (N.B.S.)—*Continued*

Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent
0.8740	75.04	0.8670	77.66	0.8600	80.20	0.8530	82.66	0.8460	85.04	0.8390	87.33
.8739	.08	.8669	.70	.8599	.23	.8529	.69	.8459	.08	.8389	.36
.8738	.12	.8668	.73	.8598	.27	.8528	.73	.8458	.11	.8388	.39
.8737	.16	.8667	.77	.8597	.30	.8527	.76	.8457	.14	.8387	.43
.8736	.19	.8666	.81	.8596	.34	.8526	.80	.8456	.18	.8386	.46
.8735	.23	.8665	.84	.8595	.38	.8525	.83	.8455	.21	.8385	.49
.8734	.27	.8664	.88	.8594	.41	.8524	.87	.8454	.24	.8384	.52
.8733	.31	.8663	.92	.8593	.45	.8523	.90	.8453	.28	.8383	55
.8732	.35	.8662	.96	.8592	.48	.8522	.94	.8452	.31	.8382	.58
.8731	.38	.8661	.99	.8591	.52	.8521	.97	.8451	.34	.8381	.62
.8730	.42	.8660	78.03	.8590	.55	.8520	83.01	.8450	.38	.8380	.65
.8729	.46	.8659	.07	.8589	.59	.8519	.04	.8449	.41	.8379	.68
.8728	.50	.8658	.10	.8588	.62	.8518	.07	.8448	.44	.8378	.71
.8727	.54	.8657	.14	.8587	.66	.8517	.11	.8447	.48	.8377	.74
.8726	.57	.8656	.17	.8586	.69	.8516	.14	.8446	.51	.8376	.78
.8725	.61	.8655	.21	.8585	.73	.8515	.18	.8445	.54	.8375	.81
.8724	.65	.8654	.25	.8584	.77	.8514	.21	.8444	.57	.8374	.84
.8723	.69	.8653	.28	.8583	.80	.8513	.25	.8443	.61	.8373	.87
.8722	.73	.8652	.32	.8582	.84	.8512	.28	.8442	.64	.8372	.90
.8721	.76	.8651	.36	.8581	.87	.8511	.32	.8441	.67	.8371	.94
.8720	.80	.8650	.39	.8580	.91	.8510	.35	.8440	.71	.8370	.97
.8719	.84	.8649	.43	.8579	.94	.8509	.39	.8439	.74	.8369	88.00
.8718	.88	.8648	.47	.8578	.98	.8508	.42	.8438	.77	.8368	.03
.8717	.91	.8647	.50	.8577	81.01	.8507	.45	.8437	.80	.8367	.06
.8716	.95	.8646	.54	.8576	.05	.8506	.49	.8436	.84	.8366	.09
.8715	.99	.8645	.57	.8575	.08	.8505	.52	.8435	.87	.8365	.13
.8714	76.03	.8644	.61	.8574	.12	.8504	.56	.8434	.90	.8364	.16
.8713	.06	.8643	.65	.8573	.16	.8503	.59	.8433	.94	.8363	.19
.8712	.10	.8642	.68	.8572	.19	.8502	.62	.8432	.97	.8362	.22
.8711	.14	.8641	.72	.8571	.23	.8501	.66	.8431	86.00	.8361	.25
.8710	.18	.8640	.76	.8570	.26	.8500	.69	.8430	.03	.8360	.29
.8709	.22	.8639	.79	.8569	.30	.8499	.73	.8429	.07	.8359	.32
.8708	.25	.8638	.83	.8568	.33	.8498	.76	.8428	.10	.8358	.35
.8707	.29	.8637	.86	.8567	.37	.8497	.79	.8427	.13	.8357	.38
.8706	.33	.8636	.90	.8566	.40	.8496	.83	.8426	.16	.8356	.41
.8705	.37	.8635	.94	.8565	.44	.8495	.86	.8425	.20	.8355	.44
.8704	.40	.8634	.97	.8564	.47	.8494	.90	.8424	.23	.8354	.47
.8703	.44	.8633	79.01	.8563	.51	.8493	.93	.8423	.26	.8353	.50
.8702	.48	.8632	.05	.8562	.54	.8492	.97	.8422	.30	.8352	.54
.8701	.52	.8631	.08	.8561	.58	.8491	84.00	.8421	.33	.8351	.57
.8700	.55	.8630	.12	.8560	.61	.8490	.03	.8420	.36	.8350	.60
.8699	.59	.8629	.16	.8559	.65	.8489	.07	.8419	.39	.8349	.63
.8698	.63	.8628	.19	.8558	.68	.8488	.10	.8418	.43	.8348	.66
.8697	.66	.8627	.23	.8557	.72	.8487	.14	.8417	.46	.8347	.69
.8696	.70	.8626	.26	.8556	.75	.8486	.17	.8416	.49	.8346	.72
.8695	.74	.8625	.30	.8555	.79	.8485	.20	.8415	.52	.8345	.75
.8694	.78	.8624	.34	.8554	.82	.8484	.24	.8414	.56	.8344	.79
.8693	.81	.8623	.37	.8553	.86	.8483	.27	.8413	.59	.8343	.82
.8692	.85	.8622	.41	.8552	.89	.8482	.31	.8412	.62	.8342	.85
.8691	.89	.8621	.45	.8551	.93	.8481	.34	.8411	.65	.8341	.88
.8690	.92	.8620	.48	.8550	.96	.8480	.37	.8410	.68	.8340	.91
.8689	.96	.8619	.52	.8549	82.00	.8479	.41	.8409	.72	.8339	.94
.8688	77.00	.8618	.55	.8548	.03	.8478	.44	.8408	.75	.8338	.97
.8687	.03	.8617	.59	.8547	.07	.8477	.47	.8407	.78	.8337	89.00
.8686	.07	.8616	.63	.8546	.10	.8476	.51	.8406	.81	.8336	.04
.8685	.11	.8615	.66	.8545	.14	.8475	.54	.8405	.85	.8335	.07
.8684	.14	.8614	.70	.8544	.17	.8474	.57	.8404	.88	.8334	.10
.8683	.18	.8613	.73	.8543	21	8473	.61	.8403	.91	.8333	.13
.8682	.22	.8612	.77	.8542	.24	.8472	.64	.8402	.94	.8332	.16
.8681	.26	.8611	.80	.8541	28	.8471	.67	.8401	.98	.8331	.19
.8680	.29	.8610	.84	.8540	.31	.8470	.71	.8400	87.01	.8330	.22
.8679	.33	.8609	.88	.8539	.35	.8469	.74	.8399	.04	.8329	.25
.8678	.37	.8608	.91	.8538	.38	.8468	.78	.8398	.07	.8328	.28
.8677	.40	8607	.95	.8537	.42	.8467	.81	.8397	.10	.8327	.31
.8676	.44	.8606	.98	.8536	.45	.8466	.84	.8396	.14	.8326	.35
.8675	.48	.8605	80.02	.8535	.49	.8465	.88	.8395	.17	.8325	.38
.8674	.51	.8604	.05	.8534	.52	.8464	91	.8394	.20	.8324	.41
.8673	.55	.8603	.09	.8533	.55	.8463	.94	.8393	.23	.8323	.44
.8672	.59	.8602	.13	.8532	.59	.8462	.98	.8392	.27	.8322	.47
.8671	.62	.8601	16	.8531	.62	.8461	85.01	.8391	.30	.8321	.50

ETHANOL BY VOLUME AT 15.56° C. FROM APPARENT SPECIFIC GRAVITY AT 20° C. (N.B.S.)—*Concluded*

Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent	Sp.Gr.	Per Cent
0.8320	89.53	0.8250	91.62	0.8180	93.58	0.8110	95.42	0.8040	97.12	0.7970	98.68
.8319	.56	.8249	.64	.8179	.61	.8109	.44	.8039	.15	.7969	.70
.8318	.59	.8248	.67	.8178	.64	.8108	.47	.8038	.17	.7968	.72
.8317	.62	.8247	.70	.8177	.66	.8107	.49	.8037	.19	.7967	.75
.8316	.65	.8246	.73	.8176	.69	.8106	.52	.8036	.22	.7966	.77
.8315	.68	.8245	.76	.8175	.72	.8105	.54	.8035	.24	.7965	.79
.8314	.71	.8244	.79	.8174	.74	.8104	.57	.8034	.26	.7964	.81
.8313	.74	.8243	.82	.8173	.77	.8103	.59	.8033	.29	.7963	.83
.8312	.77	.8242	.85	.8172	.80	.8102	.62	.8032	.31	.7962	.85
.8311	.80	.8241	.87	.8171	.82	.8101	.64	.8031	.33	.7961	.87
.8310	.83	.8240	.90	.8170	.85	.8100	.67	.8030	.36	.7960	.89
.8309	.86	.8239	.93	.8169	.88	.8099	.69	.8029	.38	.7959	.91
.8308	.89	.8238	.96	.8168	.90	.8098	.72	.8028	.40	.7958	.93
.8307	.93	.8237	.99	.8167	.93	.8097	.74	.8027	.43	.7957	.95
.8306	.96	.8236	92.02	.8166	.96	.8096	.77	.8026	.45	.7956	.97
.8305	.99	.8235	.05	.8165	.98	.8095	.79	.8025	.47	.7955	99.00
.8304	90.02	.8234	.08	.8164	94.01	.8094	.82	.8024	.49	.7954	.02
.8303	.05	.8233	.10	.8163	.04	.8093	.84	.8023	.52	.7953	.04
.8302	.08	.8232	.13	.8162	.06	.8092	.87	.8022	.54	.7952	.06
.8301	.11	.8231	.16	.8161	.09	.8091	.89	.8021	.56	.7951	.08
.8300	.14	.8230	.19	.8160	.12	.8090	.92	.8020	.59	.7950	.10
.8299	.17	.8229	.22	.8159	.14	.8089	.94	.8019	.61	.7949	.12
.8298	.20	.8228	.25	.8158	.17	.8088	.97	.8018	.63	.7948	.14
.8297	.23	.8227	.28	.8157	.20	.8087	.99	.8017	.66	.7947	.16
.8296	.26	.8226	.31	.8156	.22	.8086	96.02	.8016	.68	.7946	.18
.8295	.29	.8225	.33	.8155	.25	.8085	.04	.8015	.70	.7945	.20
.8294	.32	.8224	.36	.8154	.28	.8084	.07	.8014	.72	.7944	.22
.8293	.35	.8223	.39	.8153	.30	.8083	.09	.8013	.75	.7943	.24
.8292	.38	.8222	.42	.8152	.33	.8082	.11	.8012	.77	.7942	.26
.8291	.41	.8221	.45	.8151	.36	.8081	.14	.8011	.79	.7941	.28
.8290	.44	.8220	.48	.8150	.38	.8080	.16	.8010	.81	.7940	.30
.8289	.47	.8219	.50	.8149	.41	.8079	.19	.8009	.84	.7939	.32
.8288	.50	.8218	.53	.8148	.44	.8078	.21	.8008	.86	.7938	.34
.8287	.53	.8217	.56	.8147	.46	.8077	.24	.8007	.88	.7937	.36
.8286	.56	.8216	.59	.8146	.49	.8076	.26	.8006	.90	.7936	.38
.8285	.59	.8215	.62	.8145	.51	.8075	.29	.8005	.92	.7935	.40
.8284	.62	.8214	.65	.8144	.54	.8074	.31	.8004	.95	.7934	.42
.8283	.65	.8213	.67	.8143	.57	.8073	.33	.8003	.97	.7933	.44
.8282	.67	.8212	.70	.8142	.59	.8072	.36	.8002	.99	.7932	.46
.8281	.70	.8211	.73	.8141	.62	.8071	.38	.8001	98.01	.7931	.48
.8280	.73	.8210	.76	.8140	.64	.8070	.41	.8000	.03	.7930	.50
.8279	.76	.8209	.79	.8139	.67	.8069	.43	.7999	.06	.7929	.52
.8278	.79	.8208	.81	.8138	.70	.8068	.46	.7998	.08	.7928	.54
.8277	.82	.8207	.84	.8137	.72	.8067	.48	.7997	.10	.7927	.56
.8276	.85	.8206	.87	.8136	.75	.8066	.50	.7996	.12	.7926	.58
.8275	.88	.8205	.90	.8135	.77	.8065	.53	.7995	.14	.7925	.60
.8274	.91	.8204	.92	.8134	.80	.8064	.55	.7994	.17	.7924	.62
.8273	.94	.8203	.95	.8133	.83	.8063	.58	.7993	.19	.7923	.64
.8272	.97	.8202	.98	.8132	.85	.8062	.60	.7992	.21	.7922	.66
.8271	91.00	.8201	93.01	.8131	.88	.8061	.63	.7991	.23	.7921	.68
.8270	.03	.8200	.04	.8130	.90	.8060	.65	.7990	.26	.7920	.70
.8269	.06	.8199	.06	.8129	.93	.8059	.67	.7989	.28	.7919	.72
.8268	.09	.8198	.09	.8128	.95	.8058	.70	.7988	.30	.7918	.74
.8267	.12	.8197	.12	.8127	.98	.8057	.72	.7987	.32	.7917	.76
.8266	.15	.8196	.14	.8126	95.01	.8056	.75	.7986	.34	.7916	.78
.8265	.18	.8195	.17	.8125	.03	.8055	.77	.7985	.36	.7915	.80
.8264	.21	.8194	.20	.8124	.06	.8054	.79	.7984	.39	.7914	.82
.8263	.24	.8193	.23	.8123	.08	.8053	.82	.7983	.41	.7913	.84
.8262	.27	.8192	.25	.8122	.11	.8052	.84	.7982	.43	.7912	.86
.8261	.29	.8191	.28	.8121	.14	.8051	.86	.7981	.45	.7911	.88
.8260	.32	.8190	.31	.8120	.16	.8050	.89	.7980	.47	.7910	.90
.8259	.35	.8189	.34	.8119	.19	.8049	.91	.7979	.49	.7909	.92
.8258	.38	.8188	.36	.8118	.21	.8048	.94	.7978	.51	.7908	.94
.8257	.41	.8187	.39	.8117	.24	.8047	.96	.7977	.54	.7907	.96
.8256	.44	.8186	.42	.8116	.26	.8046	.98	.7976	.56	.7906	.98
.8255	.47	.8185	.44	.8115	.29	.8045	97.01	.7975	.58	.7905	100.00
.8254	.50	.8184	.47	.8114	.32	.8044	.03	.7974	.60		
.8253	.53	.8183	.50	.8113	.34	.8043	.05	.7973	.62		
.8252	.56	.8182	.53	.8112	.37	.8042	.08	.7972	.64		
.8251	.59	.8181	.55	.8111	.39	.8041	.10	.7971	.66		

ETHANOL BY WEIGHT FROM ETHANOL BY VOLUME AT 15.56° C. (N.B.S.) *

Per Cent by Volume	Per Cent by Weight	Difference	Per Cent by Volume	Per Cent by Weight	Difference
0	0.000	.....	50	42.487	.....
1	0.795	0.795	51	43.428	0.941
2	1.593	.798	52	44.374	.946
3	2.392	.799	53	45.326	.952
4	3.194	.802	54	46.283	.957
		.804			.962
5	3.998		55	47.245	
6	4.804	.806	56	48.214	.969
7	5.612	.808	57	49.187	.973
8	6.422	.810	58	50.167	.980
9	7.234	.812	59	51.154	.987
		.813			.993
10	8.047		60	52.147	
11	8.862	.815	61	53.146	.999
12	9.679	.817	62	54.152	1.006
13	10.497	.818	63	55.165	.013
14	11.317	.820	64	56.184	.019
		.821			.024
15	12.138		65	57.208	
16	12.961	.823	66	58.241	.033
17	13.786	.825	67	59.279	.038
18	14.612	.826	68	60.325	.046
19	15.440	.828	69	61.379	.054
		.829			.062
20	16.269		70	62.441	
21	17.100	.831	71	63.511	.070
22	17.933	.833	72	64.588	.077
23	18.768	.835	73	65.674	.086
24	19.604	.836	74	66.768	.094
		.839			.102
25	20.443		75	67.870	
26	21.285	.842	76	68.982	.112
27	22.127	.842	77	70.102	.120
28	22.973	.846	78	71.234	.132
29	23.820	.847	79	72.375	.141
		.850			.151
30	24.670		80	73.526	
31	25.524	.854	81	74.686	.160
32	26.382	.858	82	75.858	.172
33	27.242	.860	83	77.039	.181
34	28.104	.862	84	78.233	.194
		.867			.208
35	28.971		85	79.441	
36	29.842	.871	86	80.662	.221
37	30.717	.875	87	81.897	.235
38	31.596	.879	88	83.144	.247
39	32.478	.882	89	84.408	.264
		.886			.281
40	33.364		90	85.689	
41	34.254	.890	91	86.989	.300
42	35.150	.896	92	88.310	.321
43	36.050	.900	93	89.652	.342
44	36.955	.905	94	91.025	.373
		.910			.398
45	37.865		95	92.423	
46	38.778	.913	96	93.851	.428
47	39.697	.919	97	95.315	.464
48	40.622	.925	98	96.820	.505
49	41.551	.929	99	98.381	.561
		.936			.619
50	42.487	....	100	100.000	....

* *Natl. Bur. Standards* 1924, *Circ.* **19**, 18, via *Methods of Analysis, A.O.A.C.*

Ethanol by Volume at 15.56° C. from Zeiss Immersion Refractometer Reading and Refractive Index at 17.5° and 20° C. (St. John) *

Reading	Ref. Ind.	Ethanol Per Cent		Reading	Ref. Ind.	Ethanol Per Cent		Reading	Ref. Ind.	Ethanol Per Cent	
		17.5° C.	20° C.			17.5° C.	20° C.			17.5° C.	20° C.
13.2	1.33250	....	....	21.2	1.33559	4.70	5.19	29.2	1.33865	10.24	10.73
13.4	.33257	....	....	21.4	.33566	4.84	5.33	29.4	.33873	10.36	10.86
13.6	.33265	....	....	21.6	.33574	4.99	5.47	29.6	.33881	10.50	10.99
13.8	.33273	....	....	21.8	.33582	5.13	5.61	29.8	.33888	10.63	11.12
14.0	.33281	....	....	22.0	.33590	5.27	5.76	30.0	.33896	10.76	11.26
14.2	.33288	....	....	22.2	.33597	5.41	5.90	30.2	.33904	10.89	11.38
14.4	.33296	....	....	22.4	.33605	5.56	6.05	30.4	.33911	11.02	11.51
14.6	.33304	....	0.16	22.6	.33613	5.70	6.19	30.6	.33919	11.15	11.64
14.8	.33312	....	0.34	22.8	.33620	5.85	6.33	30.8	.33926	11.28	11.78
15.0	.33319	0.00	0.52	23.0	.33628	5.99	6.47	31.0	.33934	11.41	11.91
15.2	.33327	0.17	0.69	23.2	.33636	6.13	6.61	31.2	.33942	11.54	12.04
15.4	.33335	0.34	0.85	23.4	.33643	6.27	6.75	31.4	.33949	11.66	12.17
15.6	.33343	0.51	1.03	23.6	.33651	6.41	6.90	31.6	.33957	11.79	12.30
15.8	.33350	0.68	1.21	23.8	.33659	6.55	7.04	31.8	.33964	11.92	12.43
16.0	.33358	0.84	1.36	24.0	.33666	6.69	7.18	32.0	.33972	12.05	12.57
16.2	.33366	1.02	1.51	24.2	.33674	6.83	7.32	32.2	.33980	12.18	12.70
16.4	.33374	1.18	1.66	24.4	.33682	6.97	7.46	32.4	.33987	12.31	12.83
16.6	.33381	1.34	1.81	24.6	.33689	7.11	7.60	32.6	.33995	12.43	12.96
16.8	.33389	1.49	1.96	24.8	.33697	7.25	7.74	32.8	.34002	12.56	13.09
17.0	.33397	1.63	2.11	25.0	.33705	7.39	7.88	33.0	.34010	12.69	13.22
17.2	.33405	1.77	2.26	25.2	.33712	7.53	8.01	33.2	.34018	12.82	13.35
17.4	.33412	1.92	2.41	25.4	.33720	7.66	8.14	33.4	.34025	12.95	13.48
17.6	.33420	2.07	2.56	25.6	.33728	7.80	8.28	33.6	.34033	13.08	13.61
17.8	.33428	2.21	2.70	25.8	.33735	7.94	8.42	33.8	.34040	13.20	13.74
18.0	.33435	2.36	2.85	26.0	.33743	8.07	8.55	34.0	.34048	13.33	13.86
18.2	.33443	2.50	3.00	26.2	.33751	8.21	8.69	34.2	.34056	13.45	13.99
18.4	.33451	2.65	3.15	26.4	.33758	8.34	8.82	34.4	.34063	13.58	14.12
18.6	.33459	2.80	3.30	26.6	.33766	8.48	8.96	34.6	.34071	13.70	14.25
18.8	.33466	2.95	3.45	26.8	.33774	8.62	9.10	34.8	.34078	13.83	14.37
19.0	.33474	3.10	3.59	27.0	.33781	8.75	9.23	35.0	.34086	13.96	14.50
19.2	.33482	3.25	3.73	27.2	.33789	8.89	9.37	35.2	.34094	14.08	14.62
19.4	.33489	3.39	3.88	27.4	.33796	9.02	9.51	35.4	.34101	14.21	14.75
19.6	.33497	3.53	4.03	27.6	.33804	9.16	9.65	35.6	.34109	14.33	14.87
19.8	.33505	3.68	4.17	27.8	.33812	9.29	9.79	35.8	.34116	14.46	15.00
20.0	.33513	3.83	4.32	28.0	.33820	9.43	9.92	36.0	.34124	14.58	15.13
20.2	.33520	3.97	4.47	28.2	.33827	9.57	10.06	36.2	.34131	14.71	15.25
20.4	.33528	4.12	4.61	28.4	.33835	9.70	10.19	36.4	.34139	14.83	15.38
20.6	.33536	4.26	4.75	28.6	.33842	9.84	10.32	36.6	.34146	14.96	15.51
20.8	.33543	4.41	4.90	28.8	.33850	9.97	10.46	36.8	.34154	15.08	15.63
21.0	.33551	4.56	5.04	29.0	.33858	10.10	10.59	37.0	.34162	15.20	15.76

* Rearranged St. John table, based on Doroschevskii and Dvorzhanchik data (*J. Russ. Phys.-Chem. Soc.* 1908, **40**, 101). Readings of Pulfrich scale (*Z. angew. Chem.* 1899, p. 1168) were converted into refractive indices by the formula $n_D = 1.327338 + 0.000393 47X + 0.0000002 0446X^2$. Readings of other scales must be converted into refractive indices before they can be used. *Methods of Analysis*, *A.O.A.C.*, also give per cent at 18° and 19° C.

ETHANOL BY VOLUME AT 15.56° C. FROM ZEISS IMMERSION REFRACTOMETER READING AND REFRACTIVE INDEX AT 17.5° AND 20° C. (ST. JOHN)—*Continued*

Reading	Ref. Ind.	Ethanol Per Cent		Reading	Ref. Ind.	Ethanol Per Cent		Reading	Ref. Ind.	Ethanol Per Cent	
		17.5° C.	20° C.			17.5° C.	20° C.			17.5° C.	20° C.
37.2	1.34169	15.33	15.89	46.2	1.34508	20.76	21.42	55.2	1.34844	26.17	26.97
37.4	.34177	15.45	16.01	46.4	.34516	20.88	21.54	55.4	.34851	26.29	27.10
37.6	.34184	15.57	16.14	46.6	.34523	21.00	21.66	55.6	.34858	26.41	27.23
37.8	.34192	15.70	16.26	46.8	.34530	21.12	21.78	55.8	.34866	26.53	27.35
38.0	.34199	15.82	16.39	47.0	.34538	21.24	21.90	56.0	.34873	26.65	27.48
38.2	.34207	15.94	16.51	47.2	.34545	21.36	22.02	56.2	.34880	26.78	27.60
38.4	.34215	16.07	16.64	47.4	.34553	21.48	22.15	56.4	.34888	26.90	27.73
38.6	.34222	16.19	16.76	47.6	.34560	21.60	22.27	56.6	.34895	27.02	27.85
38.8	.34230	16.31	16.89	47.8	.34568	21.72	22.39	56.8	.34903	27.14	27.98
39.0	.34237	16.44	17.01	48.0	.34575	21.84	22.51	57.0	.34910	27.26	28.10
39.2	.34245	16.56	17.14	48.2	.34583	21.96	22.63	57.2	.34918	27.38	28.23
39.4	.34252	16.68	17.26	48.4	.34590	22.08	22.75	57.4	.34925	27.50	28.35
39.6	.34260	16.80	17.39	48.6	.34598	22.20	22.87	57.6	.34932	27.62	28.48
39.8	.34267	16.93	17.51	48.8	.34605	22.32	22.99	57.8	.34940	27.75	28.60
40.0	.34275	17.05	17.63	49.0	.34613	22.44	23.12	58.0	.34947	27.87	28.73
40.2	.34282	17.17	17.76	49.2	.34620	22.56	23.24	58.2	.34954	27.99	28.86
40.4	.34290	17.29	17.88	49.4	.34628	22.68	23.36	58.4	.34962	28.11	28.98
40.6	.34298	17.41	18.01	49.6	.34635	22.80	23.48	58.6	.34969	28.23	29.11
40.8	.34305	17.54	18.13	49.8	.34643	22.92	23.61	58.8	.34977	28.35	29.23
41.0	.34313	17.66	18.25	50.0	.34650	23.04	23.73	59.0	.34984	28.47	29.36
41.2	.34320	17.78	18.37	50.2	.34658	23.16	23.85	59.2	.34991	28.59	29.49
41.4	.34328	17.90	18.50	50.4	.34665	23.28	23.98	59.4	.34999	28.71	29.61
41.6	.34335	18.02	18.62	50.6	.34672	23.40	24.10	59.6	.35006	28.84	29.74
41.8	.34343	18.14	18.74	50.8	.34680	23.51	24.22	59.8	.35014	28.96	29.87
42.0	.34350	18.27	18.87	51.0	.34687	23.63	24.35	60.0	.35021	29.08	29.99
42.2	.34358	18.39	18.99	51.2	.34695	23.75	24.47	60.2	.35028	29.20	30.12
42.4	.34365	18.51	19.11	51.4	.34702	23.87	24.59	60.4	.35036	29.32	30.25
42.6	.34373	18.63	19.23	51.6	.34710	23.99	24.72	60.6	.35043	29.45	30.38
42.8	.34380	18.75	19.36	51.8	.34717	24.11	24.84	60.8	.35050	29.57	30.51
43.0	.34388	18.87	19.48	52.0	.34724	24.23	24.96	61.0	.35058	29.69	30.64
43.2	.34395	18.99	19.60	52.2	.34732	24.36	25.09	61.2	.35065	29.81	30.77
43.4	.34403	19.11	19.72	52.4	.34740	24.48	25.21	61.4	.35073	29.93	30.90
43.6	.34410	19.23	19.85	52.6	.34747	24.60	25.34	61.6	.35080	30.06	31.03
43.8	.34418	19.35	19.97	52.8	.34754	24.72	25.46	61.8	.35087	30.18	31.16
44.0	.34426	19.46	20.09	53.0	.34762	24.84	25.59	62.0	.35095	30.31	31.29
44.2	.34433	19.58	20.21	53.2	.34769	24.96	25.71	62.2	.35102	30.43	31.43
44.4	.34440	19.70	20.33	53.4	.34777	25.08	25.84	62.4	.35110	30.56	31.56
44.6	.34448	19.82	20.45	53.6	.34784	25.20	25.96	62.6	.35117	30.69	31.69
44.8	.34456	19.94	20.58	53.8	.34792	25.32	26.09	62.8	.35124	30.81	31.83
45.0	.34463	20.06	20.70	54.0	.34799	25.44	26.22	63.0	.35132	30.94	31.96
45.2	.34470	20.18	20.82	54.2	.34806	25.56	26.34	63.2	.35139	31.06	32.10
45.4	.34478	20.29	20.94	54.4	.34814	25.68	26.47	63.4	.35146	31.19	32.23
45.6	.34486	20.41	21.06	54.6	.34821	25.81	26.59	63.6	.35154	31.32	32.37
45.8	.34493	20.53	21.18	54.8	.34829	25.93	26.72	63.8	.35161	31.45	32.51
46.0	.34500	20.65	21.30	55.0	.34836	26.05	26.85	64.0	.35168	31.58	32.65

A single drop of the standard solution produces the final color change.

Calculation. Obtain the percentage by volume (P) by the following formula:

$$P = 20 - \frac{M}{2}$$

in which M is the number of milliliters of standard Mohr's salt solution required for the titration.

Examples. In wines containing from less than 5 to nearly 18%, the result varied no more than 0.02% from those by the Official Method.

It is claimed that with a battery for 10 determinations an average of 30 to 40 determinations may be made in an hour.

Raudenbush Aniline Volumetric Method.[6] The following simple method is proposed. Titrate 10 ml. of a mixture of 200 ml. of *aniline* and 300 ml. of *ethanol* with the wine added *slowly* with careful regulation of the temperature. Compare with the results obtained on standard dilutions of ethanol treated in like manner.

The results are stated to be between 0.1 and 0.2% of the truth.

Glycerol

Täufel and Thaler Phloroglucinol Test.[7] The method was devised at the University of Munich.

Apparatus. *Special Distillation Apparatus.* The small, nearly neckless distillation flask is narrower in the lower than in the upper half, which has a side tube that is fused to the inner tube of a condenser. A rubber stopper covered with tinfoil closes the neck.

Process. *Ethanol Extraction.* Place in a porcelain dish 0.5 to 2 ml. of grape wine, 2 to 5 ml. of cider. or 5 to 10 ml. of vinegar, and evaporate to dryness. If sucrose or raffinose is present, invert by heating for 10 minutes on a boiling water bath with 5 ml. of *4% sulfuric acid.* Mix with 2 g. of *40% calcium hydroxide suspension* and again evaporate to dryness. Moisten the residue with water and evaporate, repeating the treatment. Add to the residue a few drops of *ethanol* and rub up with a glass rod. Extract with 3 portions of 3 to 4 ml. of *absolute ethanol* and evaporate the extract to dryness on the water bath. Take up with 3 ml. of *1 + 1 absolute ethanol and ether*, filter, and wash with 3 ml. of the mixture. Place the filtrate in the part of the special apparatus for the purpose, which it should fill no more than half, and distil off the solvent.

Distillation with Phosphoric Acid. Add to the residue a little *powdered pumice* and 15 drops of *strongest phosphoric acid.* Distil by intermittently heating with a small flame, avoiding loss by the initial foaming, and collect a few drops of distillate in a 100 x 13 mm. test tube.

Peroxide Treatment. Add to the distillate 1 drop of *3% hydrogen peroxide solution* and 1 ml. of *hydrochloric acid.* Cool under the tap and shake vigorously for 1 minute. Destroy the excess of peroxide by adding a drop of *10% potassium iodide solution* and remove the separated iodine by a sufficient amount of *10% sodium thiosulfate solution.*

Phloroglucinol Treatment. To the solution, add 0.5 ml. of *0.15% ethereal phloroglucinol solution* and shake. The presence of glycerol in the sample is indicated by the formation of a red color due to epihydrinaldehyde. Note the intensity of the color and compare with that of a solution containing a known amount of glycerol treated in the same manner as the above distillate.

Direct Calcium Oxide Gravimetric Method for Dry Wine. This method has been official for a long time in Europe and for about forty years in the United States.

Reagents., *Milk of Lime.* Levigate 150 g. of freshly ignited CaO with 1 liter of water. Shake when used.

Ethanol-Ether Wash Liquid. Mix 100 ml. of absolute ethanol with 150 ml. of absolute ether.

PROCESS. *Lime Treatment.* Pipet 100 ml. of the sample into a porcelain dish, evaporate on the steam bath to about 10 ml., add 5 g. of fine *quartz sand*, 4 to 5 ml. of *milk of lime* for each gram of extract present, and complete the evaporation to a moist condition.

Ethanol Extraction. Add to the residue 50 ml. of *90% by volume ethanol* and rub to a uniform paste with a spatula, taking care to remove any deposit on the sides of the dish. Heat on the steam bath with stirring until boiling begins, decant on a filter, collecting the filtrate in a small flask, and wash by decantation with 10-ml. portions of hot *90% ethanol* until the filtrate measures about 150 ml.

Ethanol-Ether Extraction. Evaporate the filtrate to a sirup on a water bath heated below boiling, and transfer to a small glass-stoppered graduated cylinder by means of 20 ml. of *absolute ethanol*. Add three portions of *absolute ether*, of 10 ml. each, shaking after each addition, and allow to stand until clear. Filter, at first by decantation, wash the cylinder and paper with *ethanol-ether wash liquid*, evaporate the filtrate to a sirup, dry in a boiling water oven for 1 hour, cool in a desiccator, and weigh. Ignite below redness, weigh, and deduct the weight from the weight after drying in the water oven.

CALCULATION. Express results as grams of glycerol per 100 g. of wine.

A.O.A.C. Modification for Sweet Wine Exceeding 5 G. per 100 Ml. of Extract. Heat to boiling in a flask 100 ml. of a wine containing over 5 g. per 100 ml. of extract and add successive small portions of *milk of lime* until the color of the wine becomes at first darker, then lighter. Cool, add 200 ml. of *ethanol*, let settle, filter, and wash with *ethanol*. Combine the filtrate and washings and proceed as directed for dry wines beginning with Ethanol-Ether Extraction.

Hehner Dichromate Oxidation Volumetric Method. See Part II, D3.

Zeisel and Fanto Silver Iodide Gravimetric Method.[8] The method, devised at the Vienna Agriculture College, although more accurate than the lime method, has the disadvantage of expensive reagents and fragile apparatus. It depends on the treatment of the solution of glycerol with hydriodic acid, thereby liberating free iodine and forming isopropyl iodide which, reacting with silver nitrate, yields silver iodide thus:

$$C_3H_5(OH)_3 \rightarrow C_3H_7I \rightarrow AgI$$

APPARATUS. The *Vertical Assembly* is the von der Heide modification[9] of the Stritar apparatus.[10]

REAGENT. *Red Phosphorus Suspension*, 1 + 10. If a blank determination gives a black coloration, the substance must be discarded or purified by boiling with alkali and washing.

PROCESS. *Defecation.* Distil 100 ml. of wine with *tannin* and *barium acetate* in slight excess, using cork or all glass connections. Collect 70 ml. of liquid. Cool the residue, dilute to 50 ml. (dry wine) or 100 ml. (sweet wine) in a volumetric flask. Allow to settle until clear or filter.

Hydriodic Acid-Silver Nitrate Treatment. Introduce 5 ml. of the solution, containing not more than 0.15 g. of glycerol, into the 40-ml. flask of the assembly, add 15 ml. of *hydriodic acid* (sp.gr. 1.9), attach the cooler, the bubbling chamber containing 5 ml. of the well-shaken *red phosphorus suspension*, and the reaction chamber containing 40 ml. of *4% silver nitrate solution in 90% ethanol*. While passing a stream of washed and dried *carbon dioxide* through the apparatus at the rate of 3 drops a second, heat the flask in an oil bath to gentle boiling. During the boiling temper the condenser water by the warming bulb and hold the red phosphorus suspension in the bubbling chamber at a lukewarm temperature. Continue the boil-

ing for at least 2 hours until the precipitation is complete.

Conversion into Silver Iodide. Transfer the precipitate, consisting of a double silver salt, and the liquid into a 600-ml. beaker, rinse with about 10 volumes of water, add 5 to 10 drops of *nitric acid*, and heat on a boiling water bath for 30 minutes, thus converting the double salt into silver iodide. Collect the precipitate in a Gooch crucible or asbestos filter tube, wash, dry at 120 to 130°, cool, and weigh.

CALCULATION. Use the formula: 1 g. of $AgI = 0.3922$ g. of glycerol.

Vieböck and Brecher–von Bruchhausen Iodometric Micro Method.[11] Designed for the determination of methoxyl and ethoxyl groups, the method has been adapted by von Bruchhausen [12] to the determination of glycerol. The method has the advantage over the Zeisel and Fanto method in that (1) hydrogen phosphide and hydrogen sulfide formed by the action of hydriodic acid on sulfates do not interfere and (2) it is more exact since 6 atoms of iodine are liberated as compared with 1 by the latter method, as shown by the following reactions:

$$C_3H_7I + Br_2 \longrightarrow C_3H_7Br + IBr$$

$$IBr + 2Br_2 + 3H_2O \longrightarrow HIO_3 + 5HBr$$

$$HIO_3 + 5HI \longrightarrow 6I + 3H_2O$$

The role of red phosphorus in conjunction with elemental iodine is brought out by the following equations; neither the phosphorous acid nor the phosphoric acid interferes.

$$PI_3 \quad 3H_2O \cdot \quad 3HI + H_3PO_3$$

$$+ H_2O \cdot \quad H_3PO_4 + 2HI$$

APPARATUS. *Vieböck and Schwappach Methoxyl Micro Apparatus*, with reflux condenser and doubler.

PROCESS. *Isopropyl Iodide Formation.* As wash liquid, a suspension of red phosphorus is preferable to a solution containing 3% of thiosulfate and 1% of sodium carbonate.

Into the reaction flask of the methoxyl apparatus, measure 1.5 ml. of *hydriodic acid* (sp.gr. 1.7), 0.2 g. of *iodine*, and with cooling 0.1 g. of *red phosphorus*, then weigh (not measure) into the flask 0.3 to 0.5 g. of the solution of the unknown containing about 1% of glycerol.

Iodic Acid Formation. While passing a stream of *carbon dioxide* through the apparatus at the rate of 2 bubbles per second, boil gently for 1.5 hours, collecting the isopropyl iodide in 4 ml. of *20% sodium acetate in acetic acid*, to which have been added 3 drops of *bromine;* this forms iodic acid.

Bromine Removal. Transfer the contents of the receiver to a Sendtner flask containing 0.3 g. of *sodium acetate* completely dissolved in a little water and destroy the excess of bromine with a few drops of *formic acid.*

Iodine Titration. Add 0.3 g. of *potassium iodide* and 1 ml. of *25% sulfuric acid* and titrate the liberated iodine with *standard 0.03334 N sodium thiosulfate solution*, using *starch solution* as indicator.

CALCULATION. Use the formula: 1 ml. of 0.03334 N thiosulfate solution is equivalent to 0.51 mg. of glycerol.

Fulmer, Hickey, and Underkofler Ceric Sulfate Volumetric Method.[13] Earlier investigators employed ceric sulfate only in the absence of sugar; these workers, at the Iowa State College, adapted it for use in fermenting media containing dextrose. Sugar is determined by a modification of the Shaffer and Somogyi procedure; then in another portion both sugar and glycerol are oxidized with ceric sulfate and the glycerol is calculated, after correction for the ceric sulfate used by the sugar, by a suitable equation or from a graph.

REAGENT. *Somogyi Alkaline Copper Reagent.* Dissolve 25 g. of Na_2CO_3 and 25 g. of Rochelle salt in about 800 ml. of water. Add with stirring 40 ml. of 10% $CuSO_4 \cdot 5H_2O$ solution, then 20 g. of $NaHCO_3$, 200 g. of Na_2SO_4, and 1.5 g. of KI. Heat to boiling,

boil 30 seconds, cool, add 6 ml. of 1.0 N KIO_3, and dilute to 1 liter. Allow to settle for 1 to 2 days, then filter.

PROCESS. Dilute the sample so as to contain 0.03 to 0.50 mg. of dextrose per milliliter and determine the exact amount by the Shaffer and Somogyi procedure [14] as follows. Heat a suitable amount of the protein-free solution with a fixed amount of the *Somogyi alkaline copper reagent* in a 22 x 250 mm. Pyrex test tube for 35 minutes on a water bath instead of the 20 minutes recommended by Shaffer and Somogyi. Keep the tubes covered with glass bulbs during the heating and until the titration to prevent oxidation.

Ceric Sulfate Oxidation. Dilute the solution so that the dextrose plus glycerol does not exceed 4 mg. per ml. (better between 0.50 and 2.50 mg.). Into a 22 x 175 mm. Pyrex test tube measure exactly 5 ml. of the unknown, about 2 ml. of *1 + 1 sulfuric acid*, and 5 ml. of *0.1 N ceric sulfate in 1 M sulfuric acid.* Mix well. If less than 5 ml. of the unknown was used, dilute the mixture to 12 ml. Cover the tube with a glass bulb and heat in a boiling water bath for 1 hour.

Titration. Cool the tube in cold water for 2 hours at 20 to 25° and titrate the excess of ceric sulfate with *standard 0.1 N ferrous ammonium sulfate solution* (which is kept under an oxygen-free atmosphere), using either *erioglaucin* or *o-phenanthroline ferrous complex* (G. Frederick Chemical Co.) as oxidation-reduction indicator.

CALCULATION. Obtain the milliliters of 0.1 N ceric sulfate (V_1) used by milligrams of dextrose (D) as determined by equation (1):

$$(1)\quad \log(V_1 \times 10) = 1.091 \log(D \times 10) - 0.307$$

Subtract V_1 from the total volume of ceric sulfate used (V), thus obtaining the volume of ceric sulfate consumed (V_2) by the glycerol, then obtain the milligrams of glycerol (G) by equation (2):

$$(2)\quad \log(G \times 10) = 1.0 \log(V_2 \times 10) + 0.069$$

which simplifies to

$$(3)\quad G = 1.172 V_2$$

NOTE. In case the dextrose content exceeds 0.5 mg. prepare the reagent (0.005 N KIO_3) as follows. Dissolve in a 1-liter volumetric flask 2 g. of potassium iodide and 2 g. of sodium bicarbonate in 50 ml. of 0.1 N potassium iodate (or potassium biiodate) and dilute to volume. Place 5 ml. of this reagent in a test tube before acidifying for titration. The yield of iodine is sufficient to react with the cuprous oxide produced by 1 mg. of dextrose.

Lemoigne Nickel Dimethylglyoxime Test.[15] In studies on fermentation involving *B. lactis aerogenes* and *B. coli*, Lemoigne oxidized acetylmethylcarbinol formed from 2,3-butanediol to diacetyl with *ferric chloride*, distilled the mixture, and treated the greenish distillate with *ammonia*, *hydroxylamine hydrochloride*, and *nickel chloride*, thus obtaining a precipitate of nickel dimethylglyoxime.

Moureu and Dodé Quantitative Modification.[16] If the amount is considerable, gravimetric technique is employed; if small (less than 30 mg. per 100 ml.), the nickel dimethylglyoxime is estimated colorimetrically.

PROCESS. *Bromination.* Pipet into a small flask a few milliliters of the aqueous solution containing 0.1 to 0.2 g. of the diol, add *saturated bromine water* to a total volume of 30 ml., and seal the flask over a flame. Heat in a boiling water bath for exactly 3 minutes, cool in the dark, open, and remove the excess of bromine with *20% sodium sulfite solution.*

Oxidation and Distillation. Transfer the mixture to a 150-ml. distillation flask, add 15 ml. of *ferric chloride solution* (sp.gr. 1.45), and distil the biacetyl formed by the oxidation of the acetylmethylcarbinol into a solution containing 2 g. of *hydroxylamine hydrochloride*,

10 ml. of *20% sodium acetate solution*, and 10 ml. of *10% nickel chloride solution.*

Precipitation of Nickel Dimethylglyoxime. Heat the distillate for 1 to 2 hours on the water bath, filter the nickel salt, dry at 110°, and weigh.

CALCULATION. Multiply the weight of the nickel salt by 0.624 to obtain the weight of 2,3-butanediol. See Butylene Glycol below.

SORBITOL

Werder Dibenzalsorbitol-Hexaacetylsorbitol Test.[17] The purpose of the test is to detect fruit juice or fruit wine in grape juice or grape wine by the presence of sorbitol in the former and its absence in the latter. It responds to 10% and often to 5% of the admixture.

PROCESS. . *Clarification and Evaporation.* Shake 110 ml. of the sample with 5 to 7 g. of *animal charcoal*, heat to boiling, and filter. Distil 100 ml. of the filtrate under reduced pressure until the residue is sirupy.

Dibenzalsorbitol Formation. To the cooled residue add 4 drops of *benzaldehyde* and 1 ml. of *1 + 1 sulfuric acid*, shake, and keep in the refrigerator overnight. To the congealed mass, which forms if 10% or more of fruit wine is present, add 100 ml. of water and collect the amorphous precipitate of dibenzalsorbitol on a filter.

Hexaacetylsorbitol Formation. Heat on a boiling water bath 25 mg. of the dibenzalsorbitol precipitate with 2 ml. of *1.0 N sulfuric acid* in a small flask provided with a tube condenser, thus splitting the dibenzalsorbitol into its original constituents. Neutralize with *barium hydroxide suspension*, filter into a fractionating flask and evaporate under reduced pressure. Heat the residue on the steam bath for 1 hour with 0.2 to 0.5 ml. of *acetic anhydride* and 1 drop of *pyridine*, dilute with 2 to 5 ml. of hot water in a beaker, and allow to crystallize. The crystals of hexaacetylsorbitol melt at 98 to 99°.

Klostermann and Fachmann Direct Hexaacetylsorbitol Modification.[18] As modified the benzaldehyde treatment is omitted.

PROCESS. *Lead Clarification.* Boil 110 ml. of the sample with 5 to 7 g. of *animal charcoal* and filter. Evaporate to a sirup 100 ml. of the filtrate in a 300-ml. fractionating flask on a boiling water bath under diminished pressure. While still hot, add 150 ml. of *absolute methanol*, shake vigorously, and add slowly with shaking *lead subacetate solution* until a precipitate no longer forms that settles on cooling. Filter on a porcelain Gooch crucible, wash with *methanol*, and pass *hydrogen sulfide gas* into the filtrate until the excess of lead is precipitated. Filter, evaporate the filtrate to about 20 ml. and transfer to a 50-ml. test tube. Evaporate on a boiling water bath under diminished pressure to a thin sirup, taking care so to regulate the heat and suction as to avoid loss through foaming.

Hexaacetylsorbitol Formation. To the sirupy liquid, add a mixture of 3 ml. of *pyridine* and 9 ml. of *acetic anhydride* and heat in a boiling water bath for 45 minutes under a reflux condenser. Cool, add 20 ml. of water, neutralize with *sodium carbonate*, and extract with *ether* in a separatory funnel. Saturate the aqueous liquid with *sodium chloride* and reextract with *ether*. Wash the combined ether extracts with water, evaporate the ether, and dissolve the light yellow residue in a small amount of boiling water. While still hot, add *animal charcoal*, filter, and cool overnight in a refrigerator, thus causing the crystallization of the acetyl compound, seeding if necessary with a crystal of the pure substance. If the compound tends to separate in an oily condition, stir with a glass rod.

Determine the melting point of the crystals.

Sensitivity. The modified test detects as little as 2.5% of fruit wine in grape wine. Still greater delicacy is attainable by the microscopic method of Jahr,[19] provided one has mastered the somewhat exacting details.

Total Acids

(Total Acidity)

In the titration of wines, the presence of carbon dioxide, even in still wines, especially if new, and the intensity of the color, whether natural or artificial, are disturbing factors that must be met by special treatment and the selection of suitable indicators. Titration at a temperature just short of boiling overcomes the error due to occluded carbon dioxide and in a measure that due to amphoteric substances such as phosphates and albuminoids.

As is well known, results by one indicator may not be comparable with those by another and it is claimed that at best the titratable acidity does not parallel organoleptic sourness as consistently as does the hydrogen ion concentration.

Volumetric Methods. One of the three Official Methods [20] specifies 20 ml. of the sample and *0.05% azolitmin* as an outside indicator, a second 20 ml. of a white wine (or 5 ml. of a colored wine) and *phenolphthalein* in solution as an inside indicator, and a third (without mentioning the amount of charge) a dry powdered mixture of 1 part of *phenolphthalein* with 100 parts of *potassium sulfate* into which the wine is allowed to flow on a spot-plate from a rod. In the third method, designed for artificially colored wine, dilution of the charge with *neutral ethanol* facilitates the flow of the wine into the powder.

Process. The following instructions will be found sufficient in most cases. Pipet 20 ml. of the wine into a porcelain casserole and dilute so as to render the natural color less obscuring, heat nearly to boiling, and titrate with *0.1 N potassium hydroxide solution*, testing with one of the above *outside indicators* or delicate *litmus paper* from time to time, each portion added being such as not seriously to impair the result if the last addition is in excess of neutrality.

Calculation. Calculate the acids of wine as grams of tartaric acid per 100 ml. (1 ml. of 0.1 *N* NaOH = 0.0075 g. of $C_4H_6O_6$) and of cider as grams of malic acid per 100 ml. (1 ml. of 0.1 *N* NaOH = 0.0067 g. of $C_4H_6O_5$).

Vièles Iodometric Method.[21] The method depends on the following reaction:

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 +$$

Reagent. *Iodate-Iodide Reagent.* Dissolve 7.6 g. of KIO_3, 50 g. of KI, and 49.6 g. of $Na_2S_2O_3 \cdot 5H_2O$ in water and dilute to 1 liter. Mix 5 ml. with 5 ml. of 0.1 *N* H_2SO_4 and titrate against 0.05 *N* iodine solution, using starch solution as indicator.

Process. *Titration of Reagent.* Titrate 5 ml. of the *iodate-iodide solution* with the *standard 0.05 N iodine solution*, using *starch solution* as indicator; the number of milliliters required equals V. Titrate another 5 ml. of the *iodate-iodide solution* mixed with 5 ml. of *0.1 N sulfuric acid* with *standard 0.05 N iodine solution*, using *starch solution* as indicator; the number of milliliters required equals v'.

Titration of Sample. Place 5 ml. of the sample (freed from carbon dioxide if a new wine) and 5 ml. of the *iodate-iodide solution* in a 12- to 15-ml. glass-stoppered flask, shake, add freshly distilled water, stopper, and let stand at room temperature 24 hours. Transfer to a wide-mouthed precipitating jar, rinse with 5 ml. of water, and add 5 ml. of *starch solution*. Titrate with the *standard 0.05 N iodine solution* to the first appearance of a blue color; the number of milliliters required equals v.

Calculation. Obtain the acidity in grams of sulfuric acid per liter (A) from the following formula:

$$\frac{4.9}{V - v'} = p$$

and

$$\frac{4.9}{V} = g \quad \text{also} \quad A = p - gv$$

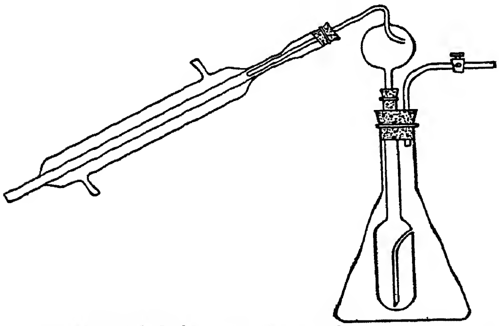
in which v is the number of milliliters of io-

dine solution required, p and g are standardization constants of the iodate-iodide and iodine solutions respectively, and V and v' are as given above.

NOTE. The results are slightly higher than by the usual method using litmus as indicator.

VOLATILE ACIDS

The volatile acids of wine consist chiefly of acetic acid formed in small amounts during manufacture and storage and in considerable



Courtesy of Methods of Analysis, A.O.A.C. 1935, p. 167

FIG. 146. Modified Hortvet-Sellier Volatile Acids Assembly.

amounts during spoilage. Minute amounts of other volatile acids, such as formic, butyric, and propionic, may be present. The total volatile acids of wine are determined by distillation with steam and titration of the distillate, or indirectly by evaporation, titration of the non-volatile acids, and subtraction from the total acids.

Official A.O.A.C. Steam Distillation Methods. Method I.[22] APPARATUS. A train consisting of (1) a *steam-generating flask* containing boiled water, (2) a *distilling flask* with a steam inlet tube long enough to dip well beneath the wine and an outlet tube passing just through the stopper, also if desired a funnel tube with a cock for adding the wine, (3) a *condenser*, and (4) a *receiver* into which the outlet tube connecting with the condenser dips beneath a small amount of water.

PROCESS. *Distillation.* Raise the stopper with tubes from the distilling flask, introduce 50 ml. of the sample by a pipet, or introduce the sample through the funnel tube, and close. If necessary to prevent foaming, add a little *tannic acid* or a small piece of *paraffin.* Seat the stopper, pass steam into the distilling flask, heating at the same time with a Bunsen burner. Regulate the steam current and heat so that the bulk of liquid in the distilling flask does not increase, but rather is held constant between 25 and 50 ml. After 200 ml. have passed over into the receiver, remove the heat and titrate the distillate with *standard 0.1 N sodium hydroxide solution,* using *phenolphthalein indicator.*

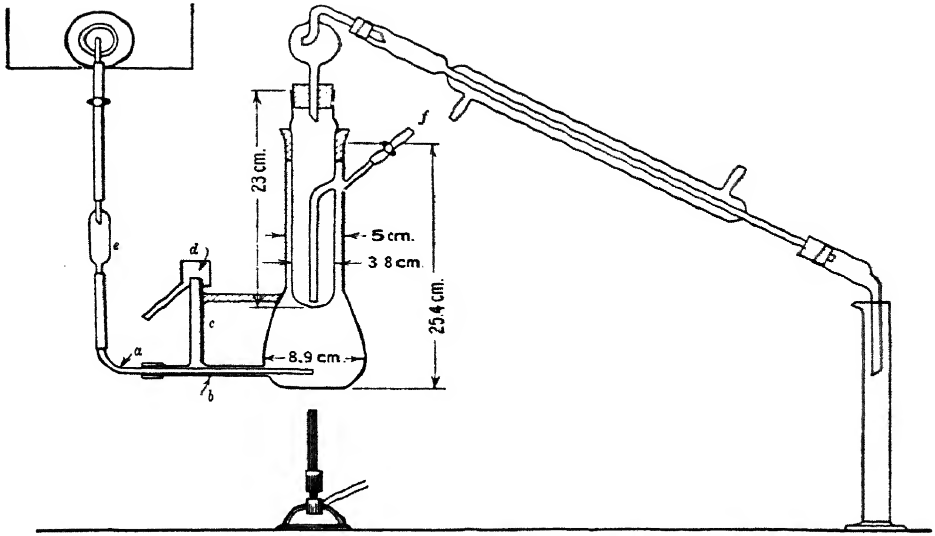
CALCULATION. Calculate the result as grams of acetic acid per 100 ml.; 1 ml. of 0.1 N NaOH = 0.006 g. of $C_2H_4O_2$.

Method II.[23] APPARATUS. (1) *Modified Hortvet-Sellier Assembly*[24] (Fig. 146).

(2) The *Hortvet-Sellier-Gore Assembly*[25] (Fig. 147) is suited for the routine analysis of numerous samples. The outer flask is of copper connected by b with the constant level device (d) for regulating the supply of water freed from carbon dioxide by distillation and aeration.

PROCESS. *Distillation.* Introduce 150 ml. of freshly boiled water into the outer flask, attach the inner tube containing a little *tannic acid* or a bit of *paraffin* if required, and make sure that the section of rubber tubing or the carefully bored rubber stopper forms a tight joint. Add 10 ml. of the sample through a funnel tube, connect with the spray trap and condenser, heat to boiling, and continue the boiling until 80 ml. of distillate have passed into the receiver. Without stopping the distillation, remove the 80 ml. to a beaker, boil for 30 seconds if the wine is new or carbonated, and cool.

Titration. Titrate with *standard 0.1 N sodium hydroxide solution,* using *phenol-*



Courtesy of U. S. Dept. Agr., Bur. Chem. 1909, Circ. 44

FIG. 147. Hortvet–Sellier-Gore Volatile Acids Assembly.

phthalein indicator, then add and titrate each 10 ml. of distillate as collected, continuing until 1 drop of standard alkali is sufficient for neutralization. Cool, allow water to suck back into the inner tube for rinsing.

CALCULATION. See Method I.

Cruess and Bettoli Double Titration Volumetric Method.[26] This method was devised by two recognized authorities on the production and analysis of wine.

PROCESS. *First Titration.* Decolorize 75 ml. of the sample by shaking with *carbon dioxide-free bone black*, filter, and titrate 20 ml. of the filtrate with *standard 0.1 N sodium hydroxide solution*, using *phenolphthalein indicator*.

Second Titration. Pipet another 20-ml. aliquot of the decolorized wine into a 200-ml. Erlenmeyer flask, add 2 g. of *sodium chloride*, and evaporate rapidly until a copious amount of the salt separates and the liquid begins to spatter. Add 20 ml. of water and repeat. Again add 20 ml. of water and repeat a second time, then titrate with *standard 0.1 N alkali*.

CALCULATION. Multiply the difference between the two titrations by 0.03 to obtain the grams of volatile acids per 100 ml.

Continental Methods. Recognizing the importance of volatile acidity as a measure of acetic acid content and consequently of soundness, European wine chemists have proposed various procedures. The German Official Method has long been essentially the same as Method I of the A.O.A.C., except that exactly 200 ml. of the distillate are collected and titrated. Such a method is distinctly conventional. The distillate, as Windisch[27] and others have claimed, does not contain all the acetic acid and, as Kulisch[28] and Lamberti[29] insist, may contain in addition to acetic acid more or less lactic acid which is slowly volatilized during the latter stages.

The French Official Method depends on evaporation *in vacuo* of 100 ml. of the wine at room temperature, titrating the residue, and deducting the non-volatile acids from the total

acids. In the similar Roos-Mestrezat method, 5 to 10 ml. of the sample are evaporated at 80° under a pressure of 65 to 70 cm. of mercury, the residue is taken up in water, the evaporation is repeated, and finally the residue, taken up in 40 to 50 ml. of water, is titrated. Miss Pujo [30] regards this method as more accurate than the distillation methods of Blarez and of Duclaux, and the French Official Method, all of which yield lower results, also the Pasteur-Gayon method which yields higher results. Mrs. Lamberti, on the other hand, reiterates the findings of earlier chemists that the higher results are due to entrainment of lactic acid. She found that the entrainment error is slight in the Duclaux-Mathieu method in which 100 ml. of wine, diluted with 10 ml. of water, are steam-distilled, 100 ml. of the distillate are titrated, and the result is multiplied by 0.8.

Organic Volatile Acids

(Volatile Acidity Exclusive of Sulfur Dioxide)

Sauternes and other sulfured wines contain sulfur dioxide which, in the determination of true or organic volatile acids by two tentative A.O.A.C. methods (above), are removed previous to distillation (Method II, below) or are determined in the distillate after titration and deducted from the result (Method I).

I. Iodometric Method. Process. Immediately after the titration of the distillate by the above methods, cool in an ice bath and add rapidly 5 ml. of *1 + 3 sulfuric acid* to 100 ml. of liquid, then titrate with *0.1 N iodine solution*, using several milliliters of *starch indicator*.

Calculation. Deduct the number of milliliters of 0.1 N sodium hydroxide required from that required in the previous titration and multiply the difference by 0.006, thus obtaining the grams of acetic acid.

II. Barium Hydroxide Volumetric Method. Process. To 50 ml. of the sample in a 100-ml. volumetric flask, if a white wine, add 2 or 3 drops of *phenolphthalein indicator* and titrate with a *saturated barium hydroxide solution* to a decided pink color; if a red wine, add the *barium hydroxide solution* to pH 8, as determined by an outside indicator. Allow to stand 30 minutes, maintaining the alkaline reaction, make up to the mark, mix, and filter rapidly on a pleated paper (Whatman No. 2). Pipet 20 ml. of the filtrate into the inner tube of the assembly, add 1 ml. of *1 + 3 sulfuric acid*, distil 100 ml., and titrate the distillate with *0.1 N sodium hydroxide solution*, using *phenolphthalein indicator*.

Calculation. Multiply the number of milliliters of alkali required by 0.006, thus obtaining the grams of acetic acid.

Note. Marcille [31] criticizes the procedure of Mathieu based on the Sadler reaction. He corrects the volatile acidity of sulfured wine, expressed in terms of sulfuric acid, by subtracting the free and 0.7 times the combined sulfurous acid, or in grams per 100 milliliters, by subtracting 1.55 times the free sulfurous acid and 1.08 times the combined sulfurous acid. For example: from 1.80 g. per 100 ml. volatile acidity uncorrected in a wine containing 0.8 and 1.12 g. per ml. respectively of free and combined sulfurous acid, the deduction is $(0.08 \times 1.55) + (1.12 \times 1.08) = 1.345$ and the volatile acidity corrected is $1.8 - 1.35 = 0.45$ g. per ml.

Non-Volatile Acids

Official A.O.A.C. Method. Obtain the non-volatile acids by difference, subtracting from the content of total acids the content of volatile acids, both calculated as tartaric acid, by multiplying the acidity expressed as acetic acid by the factor 1.25.

Total Tartaric Acid

See also Part II, D2.

Hartmann and Eoff Ethanol Precipitation Volumetric Method.[32] Process. *Precipita-*

tion. Add to 100 ml. of the sample an amount of *1.0 N sodium hydroxide solution* sufficient to neutralize the acidity, as obtained in the titration of the total acids above, or by actual titration of the portion measured for analysis. By this neutralization, the disturbing influence of phosphoric acid and perhaps other mineral acids is overcome. Evaporate to about 100 ml. if the volume of liquid added exceeds 10 ml. For each milliliter of alkali required for neutralization, add exactly 0.075 g. of *powdered tartaric acid* of known purity, dried for 2 hours at 100°, stirring until dissolved. Add 2 ml. of *glacial acetic acid*, 15 g. of *potassium chloride*, stirring until dissolved, and 15 ml. of *ethanol*, continuing the stirring vigorously until potassium bitartrate begins to precipitate; then store in a refrigerator at 15 to 18° for at least 15 hours.

Decant the liquid on a Gooch crucible with a thin layer of asbestos or on a Büchner funnel fitted with a paper. Wash three times with a solution of 15 g. of *potassium chloride* in a mixture of 20 ml. of *ethanol* and 100 ml. of water, limiting the total used to 20 ml. Return the precipitate, together with asbestos or paper, to the beaker, rinse with 50 ml. of hot water, and heat to boiling.

Titration. Titrate the hot solution with *0.1 N sodium hydroxide solution*, using *phenolphthalein indicator*, adding to the number of milliliters required for the titration 1.5 ml. as a correction for the solubility of the potassium bitartrate.

CALCULATION. Obtain the total weight of tartaric acid in the precipitate by multiplying the corrected number of milliliters by the factor 0.015, then by subtracting from the product the weight of tartaric acid added obtain the weight in the 100 ml. of the sample. Calculate as grams per 100 ml. of the wine.

Kling Calcium Racemate Volumetric Method.[33] The original method is briefly as follows: dilute to 100 ml. 25 ml. of the sample containing 3 to 4 g. per liter of *d*-tartaric acid, add 25 ml. of *1.0% Rochelle salt* (free from *d*-acid) and 20 ml. of *3% calcium acetate solution.* Collect the precipitate on a filter, wash, and dissolve in 20 ml. of *4% hydrochloric acid*, dilute to 150 ml., then add 40 ml. each of *10% sodium acetate solution* and *1% calcium acetate solution.* Heat to boiling, filter, wash, and redissolve the precipitate in *10% sulfuric acid.* Titrate while boiling with *potassium permanganate solution* standardized against pure bitartrate.

Kling and Florentin Calcium Racemate Volumetric Method. Following Pasteur,[34] who describes a racemate method said to yield accurate results, Kling[35] and Kling and Florentin,[36] both of the Paris Municipal Laboratory, developed a racemate method employing calcium acetate as the precipitant.

Sémichon and Flanzy Modification.[37] The improvements over the original method are (1) substitution of calcium sulfate for calcium acetate, (2) delay of 5 to 10 minutes after adding the ammonium tartrate before adding the calcium sulfate, and (3) precipitation while hot.

PROCESS. *Precipitation with Calcium Sulfate.* To 5 ml. of the sample add 5 ml. of *2% ammonium l-tartrate solution.* After 10 minutes, add 50 ml. of *saturated calcium sulfate solution* and heat to boiling, then allow to stand overnight. Filter and wash with 4 portions of 5 ml. each of cold water.

Titration. Suspend the precipitate in 100 ml. of water, add 5 ml. of *50% sulfuric acid*, heat to boiling, and titrate during boiling with *0.4% potassium permanganate solution* to a faint rose color.

CALCULATION. Obtain the content of tartaric acid in terms of grams of potassium bitartrate per liter (T) from the formula

$$T = \frac{P \times B \times 1000}{5}$$

in which P is the number of milliliters of permanganate solution required in the titration

and B is the grams of tartaric acid equivalent to 1 ml. of the permanganate solution.

François and Lormand Calcium Tartrate Gravimetric Method.[38] Reagent. *Calcium Acetate Solution*, concentrated. Dissolve 100 g. of precipitated $CaCO_3$ and 150 ml. of glacial acetic acid in water and dilute to 1 liter.

Process. *Defecation*. Pipet 125 ml. of the sample into a 250-ml. volumetric flask, add 25 ml. of *20% normal lead acetate solution*, and mix, then add immediately 25 ml. of *40% crystalline sodium carbonate solution*, dilute to the mark, shake, and allow to stand 2 hours. Filter on a dry pleated paper.

Calcium Tartrate Precipitation. Pipet 200 ml. of the alkaline lilac-colored filtrate into a 400-ml. Erlenmeyer flask, add 4 ml. of *glacial acetic acid*, which renders the solution acid and produces a rose coloration, then add 40 ml. of *calcium acetate solution*, followed by 50 ml. of *ethanol*. After mixing, seed with a few minute crystals of *calcium tartrate*, introduced from the end of a moistened glass rod, and after 1 hour add 60 ml. of *ethanol*, mix, and allow to stand 48 hours.

Filtration. Collect the crystals on a weighed Gooch crucible or weighed filter, wash with a little *ethanol*, allow to dry in the air at room temperature, and weigh.

Calculation. To obtain the weight of tartaric acid in 100 ml. of the wine, multiply the weight of the crystals of calcium tartrate by 0.5769.

Example. From a mixture of pure tartaric acid and potassium sulfate 99.14% of the acid was recovered.

Berg and Schmechel Acetic Acid-Acetate Volumetric Method.[39] The method originated at the Hamburg Institute of Hygiene.

Process. *Precipitation*. Pipet 50 ml. of the sample into a 250-ml. beaker, add 15 g. of finely ground *potassium chloride*, and dissolve as completely as possible by stirring and, if necessary, gentle warming; a small amount remaining undissolved is, however, not detrimental. Add 2 ml. of *glacial acetic acid* and 1 ml. of *1 + 4 potassium acetate solution*. Cool to 0° or below in a salt-ice mixture, thus precipitating the potassium chloride which separates in part before the cooling and in larger amount as the temperature falls. Add with constant stirring 100 ml. of *ethanol*, and cool to 0° or below, taking care that at no time the temperature exceeds 5°. Keep for a short time in the freezing mixture, then allow to stand overnight in a rather cool room preferably with refrigeration.

Filtration. Using a Jena glass filter crucible 1 G4 and gentle suction, transfer the precipitate to the crucible while in suspension brought about by vigorous stirring before each pouring, thus forming alternate layers in the crucible of coarse and fine crystals which are easily washed. Employ full suction only during the transfer of the last portion, then rinse the beaker successively with *ethanol*, *1 + 1 ethanol-ether mixture*, and *ether*, then again with *ethanol* (12 to 15 ml.), following the same technique as in the transfer of the precipitate, and using full suction only for short periods, thus avoiding the formation of crusts on the surface.

If it is desired to test the purity of the precipitate by the usual methods, the filtration may be interrupted and the crucible and contents dried and weighed; then the filtration is completed and the remaining portion of the precipitate is weighed.

Titration. Place the crucible and contents in the beaker previously used and dissolve the precipitate by heating to boiling with about 75 ml. of water and titrate while hot with *0.1 N standard sodium hydroxide solution*, using *litmus paper* as indicator. The end-point is reached when a drop of the liquid delivered from a glass rod to the test paper produces a change from red to blue which occurs when 1 or 2 drops are added after the violet neutral point is reached.

Calculation. Multiply the number of milliliters of 0.1 N alkali used by 0.3, thus obtaining the grams of tartaric acid per liter.

EXAMPLES. Various red and white wines 0.168 to 0.282% of tartaric acid by the new method, 0.150 to 0.254% by the Official German Method. Red Burgundy 0.205, same plus 0.4% of malic acid 0.228, same plus 0.2% of succinic acid 0.205, same plus 0.2% of citric acid 0.204% of tartaric acid respectively. Sweet wines: Sherry 0.099 and 0.069, Port 0.120 and 0.093, Malaga 0.174 and 0.146, Muscatel 0.171 and 0.141, and cider 0.101 and 0.109% of tartaric acid respectively by the new and German official methods.

FREE TARTARIC ACID

Hartmann and Eoff Calculation Method.[40] Calculate from data secured in other determinations as follows.

Let A = percentage of total tartaric acid divided by 0.015, B = total alkalinity of the ash, C = alkalinity of the water-soluble ash, D = the alkalinity of the water-insoluble ash; then if A is greater than B

$$\text{Free tartaric acid} = 0.015(A - B)$$

If A equals B, or is less than B, but is greater than C

$$\text{Free tartaric acid} = 0$$

POTASSIUM BITARTRATE

(Cream of Tartar)

Hartmann and Eoff Calculation Methods.[40] For significance of the letters, see Free Tartaric Acid above.

If A is greater than or equals B, or is less than B but is greater or less than C

$$\text{Potassium bitartrate} = 0.0188C$$

If A is less than C

$$\text{Potassium bitartrate} = 0.0188 \times A$$

CITRIC ACID

See also Part II, D2 and G1.

Kunz Pentabromacetone Gravimetric Method.[41] Twenty years after its discovery, Kunz applied the Stahre reaction to the quantitative determination of citric acid in wine, also to its detection in milk and fruit products.

PROCESS. *Oxidation.* Evaporate 100 ml. of the sample in a porcelain dish to 10 to 20 ml., cool, add 2 ml. of *dilute sulfuric acid*, then 20 ml. of *bromine water* (white wines) or a sufficient amount to form a distinct yellow color at the edges (red wines). Transfer to a 100-ml. volumetric flask, make up to the mark, and filter. Pipet 50 ml. of the filtrate into an Erlenmeyer flask, add 10 ml. of *1 + 1 sulfuric acid* and 3 ml. of *15 + 40 potassium bromide solution*, then shake and heat for 5 minutes at 50° in a water bath. Add to the warm liquid 25 ml. of *5% potassium permanganate solution* with vigorous shaking, keeping the temperature below 55°. Allow to stand until the manganic hydroxide, $Mn(OH)_4$, begins to settle, then shake until most of the precipitate dissolves. Dissolve the remainder and remove the excess of bromine by adding dropwise *concentrated ferrous sulfate solution acidulated with sulfuric acid.*

Pentabromacetone Precipitation. Allow the solution to cool with occasional shaking. When the dense white precipitate has become crystalline and has settled completely, collect it in a tared filter dried over sulfuric acid or a Gooch crucible, wash with *water slightly acidulated with sulfuric acid*, or *1% acid* if the precipitate tends to pass through the filter. If the precipitate separates as oily drops, allow to stand until these form a crystalline deposit, often discolored by iron or manganese.

Dry at room temperature in a vacuum desiccator, protected from the light, and weigh.

CALCULATION. Multiply the weight of pentabromacetone by 0.464 to obtain the weight of citric acid containing 1 molecule of water.

EXAMPLES. Pure wine contains only traces of citric acid; grape must contains none.

Reichard Pentabromacetone Gravimetric Method.[42] The

system is not allowed to exceed 5°; furthermore the bromine-citric acid ratio and the bromide-citric acid ratio are kept respectively at not less than 2 and 3.

Reagents. *Denigès Reagent.* Dissolve 5 g. of HgO in 20 ml. of H_2SO_4 and dilute to 100 ml. with water.

Bromide-Bromate Solution. Dissolve 8.5 g. of KBr and 2.5 g. of $KBrO_3$ in water and dilute to 100 ml.

Process. A. Preliminary Test. Shake in a test tube 10 ml. of the sample with 2 ml. of *Denigès reagent* and as much *animal charcoal* as can be held on the end of a penknife, then filter through a dry pleated paper. Heat the filtrate nearly to boiling and add *saturated potassium permanganate solution* dropwise until manganese dioxide precipitate separates. After a few minutes add *perhydrol* until the precipitate dissolves. If no precipitate or only a trace remains undissolved, citric acid is absent or present only in small amount. A turbidity followed by precipitation or a dense flocking shows the presence of citric acid in corresponding amounts as follows:

1. Milk-white turbidity with bluish opalescence which does not deposit in a minute indicates normal content up to 400 mg. per liter.

2. More pronounced opaque turbidity depositing in a few minutes indicates abnormal content up to 500 mg. per liter.

3. Dense white clouds appearing with the first drop of permanganate and quickly settling and filling half the "cup" of the test tube indicate 1 g. per liter.

4. Still denser turbidity, separating as flocks and filling completely the "cup" of the test tube, indicates more than 2 g. per liter.

B. Direct Bromination (For Dry Wines with Not More than 5 Grams of Sugar per Liter). *Bromide-Bromate Treatment.* Pipet into a porcelain dish 100 ml. of wine, 50 ml. of liquids containing over 1 g. per liter of citric acid, or 10 to 20 ml. of berry wine containing over 2 g. of citric acid per liter. Evaporate to about 10 ml. on the water bath and transfer to a 50-ml. volumetric flask. Add 10 ml. of *1 + 1 sulfuric acid* and sufficient *bromide-bromate solution* to impart a distinct orange to white wine (about 5 ml.) or yellow-brown to red wine (5 to 10 ml.) color. Complete the volume, mix, and filter on a dry paper.

Measure with a safety pipet 20 ml. of the golden yellow filtrate into an Erlenmeyer flask, add an excess (2 to 5 ml. according to the intensity of the Denigès reaction) of *50% potassium bromide solution* and a drop of *saturated ferric chloride solution*, mix, and cool to 5°, then add 8 to 10 ml. dropwise of *saturated potassium permanganate solution* from a buret with continual stirring until a dark brown color persisting several minutes is formed. Stopper with a cork and keep in a cold water bath for some minutes until the dark brown color changes to golden yellow. Repeat the addition of the permanganate to a dark color and after the color changes to golden yellow continue the oxidation until a turbidity, then a separation and clumping of the pentabromacetone, and finally a separation of manganese dioxide takes place, the total volume of permanganate required being about 40 ml. Let stand 1 hour, dissolve the dioxide in *50% potassium bromide solution*, using in addition for large amounts *acidulated ferrous ammonium sulfate solution*, and set aside to settle. Filter on a weighed Gooch or Neubauer crucible, wash with 5 ml. of cold water, and dry for 2-hour periods over sulfuric acid in a vacuum desiccator to constant weight. Dissolve the precipitate by treatment with *ether*, *ethanol*, and finally with 10 ml. of warm water, dry as before, and weigh. The loss in weight is the weight of the pentabromacetone.

Calculation. Multiply the weight of pentabromacetone by 1.16 to obtain the weight of citric acid in 100 ml. of the sample.

C. Bromination after Fermentation of Sugar (For Wines and Liquids Contain-

ing More than 5 Grams of Sugar per Liter). *Treatment with Yeast.* Dealcoholize 100 ml. of the sample by evaporation in a porcelain dish to half its volume. Cool, transfer to a large flask, add a few milliliters of *liquid yeast*, preferably a pure culture, dilute if necessary to a sugar content of not more than 10%, and allow to ferment in a warm place. When fermentation ceases, evaporate in a porcelain dish to 10 ml., and transfer to a 50- or 100-ml. volumetric flask. Brominate with *bromide-bromate solution* and *potassium permanganate solution* and obtain the weight of the pentabromacetone as under B.

D. Bromination after Barium Precipitation (For Liquids Containing More than 5 Grams of Sugar per Liter; also Sugar-Free Liquids Containing Small Amounts of Citric Acid). *Barium Precipitation.* Pipet into a porcelain dish 100 or 50 ml. of the sample according as the Denigès reaction shows more than 1 or 2 g. respectively of citric acid. Add *ammonium hydroxide* to weak alkaline reaction, then twice as many milliliters (usually 10 to 20) of *10% barium chloride solution* as the content of total acids calculated as tartaric acid. Evaporate to 20 ml., neutralize with *ammonium hydroxide,* transfer to a centrifuge tube, and adjust to about 50% by volume ethanol content. Cool quickly, filter or decant, and wash the precipitate twice with *50% ethanol.* Transfer to a porcelain dish, dealcoholize, then transfer to a 50-ml. volumetric flask, using 15 ml. of *1 + 1 sulfuric acid* for rinsing, treat with *bromide-bromate solution* and proceed as under B.

Lactic Acid

See also Part I, C6*b;* Part II, C3 and G1.

Mösslinger Barium Chloride Volumetric Method.[43] The method depends on the precipitation of lactic acid as the barium salt in a 70% ethanolic solution in which the barium salts of tartaric, malic, citric, and succinic acids are soluble. As modified by Bonifazi [44] in Italy and by Ferré [45] in France, it is essentially as follows.

Process. Carefully neutralize 25 ml. of the sample in a 150-ml. volumetric flask with *saturated barium hydroxide solution*, then add 2.5 ml. of *10% barium chloride solution.* Dilute to the mark with *ethanol,* shake, allow to settle, filter through a dry paper, evaporate 60 ml. (= 10 ml. of the sample), and ignite. Take up in 20 ml. of *0.1 N hydrochloric acid* and back-titrate. The result calculated to milliliters of normal acid represents the sum of the lactic acid and the volatile acids. Deduct the number of milliliters corresponding to the volatile acids determined by a suitable method and multiply the difference by 0.09 to obtain the grams of lactic acid.

Michel Modification.[46] Certain defects of the original method and previous modifications are overcome by the following procedure.

Process. *Barium Chloride Precipitation.* Place 20 ml. of the sample in a 110-ml. volumetric flask, neutralize to *phenolphthalein* with *saturated barium hydroxide solution,* then add 2 ml. of *10% barium chloride solution,* followed by *ethanol* to about 100-ml. Again neutralize to a rose tint and make up to the 110-ml. mark. Shake and let stand for 3 hours to insure precipitation of the barium succinate, centrifuge or filter, wash, and remove the excess of barium from the solution by a current of *carbon dioxide* or simple aeration through a narrow tube. An excess of the gas is indicated by the coloration of the indicator, but continue the flow of gas until the precipitate flocks, then filter.

Ignition. Pipet 80 ml. of the clear solution into a porcelain dish, cautiously evaporate the ethanol on a water bath, add 2 ml. of an approximately *1.0 N potassium sulfate solution,* and continue the evaporation to dryness, then ignite.

Titration. To the calcined residue, add an excess of *0.2 N hydrochloric acid* and titrate back with *0.2 N sodium hydroxide solution.*

Determine the volatile acids as in the original method.

CALCULATION. Calculate the number of milliliters of lactic acid per liter (L) in terms of N acid from the equation

$$L = (N \times 13.75) - V$$

in which N is the number of milliliters of N acid required for the titration of the calcined residue and V is the number of milliliters of N alkali required for the titration of the volatile acids.

NOTE. Fabre and Bremond [47] endorse a procedure essentially the same as the Michel modification.

Sémichon and Flanzy Chromic Trioxide Volumetric Method.[48] Lactic acid is oxidized to acetic acid which is distilled and titrated. Tartaric and malic acids are oxidized to carbon dioxide and water.

PROCESS. *Calcium Hydroxide Precipitation.* To 50 ml. of the sample, add an excess of *calcium hydroxide*, reflux 1 hour, and evaporate to about 25 ml. Cool, add an excess of *tartaric acid,* and filter after 2 hours into a 200-ml. Kjeldahl flask, then wash 12 times with water up to about 50 ml.

Oxidation. Steam-distil to remove the alcohols, aldehydes, and volatile acids. Cool the residue, add 1.5 g. of *chromic trioxide* and 2 ml. of *sulfuric acid* (sp.gr. 1.71), reflux on a boiling water bath for 1 hour, and again distil.

Lactic Acid Titration. Titrate the distillate with *lime water,* using *litmus indicator* in the usual manner. If desired, correct for entrained lactic acid (0.76 to 1.22% of the total amount) as described by the same authors,[49] but this is usually not necessary.

CALCULATION. One molecule of acetic acid corresponds to one molecule of lactic acid.

NOTE. von Fellenberg [50] determines the total organic acids (tartaric, malic, succinic, and lactic) by extraction with ether after decolorization with bone black, and titration with 0.1 N sodium hydroxide solution. Lactic acid is determined in the titrated mixture by the silver salt method. The results, although accurate for white wine and cider, are somewhat high for red wine.

SUCCINIC ACID

Sémichon and Flanzy Chromic Acid Gravimetric Method.[51] A mixture of chromic and sulfuric acids destroys tartaric and malic acids but does not attack succinic acid.

PROCESS. *Chromic Acid Treatment.* Pipet 50 ml. of the sample into a 200-ml. Kjeldahl flask placed in cold water, and add 1.5 g. of *powdered chromic acid* and 2 ml. of *sulfuric acid* (sp.gr. 1.71). Reflux on a boiling water bath for 1 hour, during which time all the organic acids other than succinic are oxidized to carbon dioxide and water or to acetic acid. Steam-distil to remove the acetic acid.

Reduction of Chromic Acid. After cooling, add to the residue in the flask *ammonium sulfhydrate* and precipitate the reduced chromic acid with *ammonium hydroxide.* Boil until the odor of ammonia disappears, filter, and wash with water, thus obtaining a solution containing only ammonium sulfate and ammonium succinate.

Precipitation of Ammonium Sulfate. Evaporate the solution to about 1 ml. and add 50 ml. of *ethanol,* thus precipitating all the ammonium sulfate. Filter, wash with ethanol, remove the ethanol from the filtrate by distillation, evaporate to dryness, and weigh the ammonium succinate. Test for sulfuric acid. If present, remove by precipitation with *barium hydroxide* and correct accordingly.

CALCULATION. Ammonium succinate is a stable crystalline salt containing no water of crystallization. Obtain the grams of the acid in 1 liter (S) from the equation

$$S = W \times \tfrac{118}{152} \times 20$$

in which W is the weight of the salt.

NOTE. In the same portion may be determined successively:

(1) *Volatile acids* by steam-distillation in the usual manner.

(2) *Lactic acid* by the method of Sémichon and Flanzy above (which see).

(3) *Succinic acid* by the method described herewith.

BUTYLENE GLYCOL, ACETYL-METHYL CARBINOL, AND DIACETYL

Kniphorst and Kruisheer Combination Method.[52] The three substances of the group are present not only in wine, but also in butter, coffee, and other foods, contributing to the desirable flavor. The nature of the oxidative changes from butylene glycol to diacetyl, with acetyl-methyl carbinol intermediate, is obvious from the formulas:

$$CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$$
Butylene glycol (Butanediol)

$$CH_3 \cdot CO \cdot CHOH \cdot CH_3$$
Acetyl-methyl carbinol

$$CH_3 \cdot CO \cdot CO \cdot CH_3$$
Diacetyl

In the original form, the Lemoigne[53]-van Niel[54] gravimetric method employing nickel chloride ($NiCl_2 \cdot 6H_2O$) was devised for the determination of diacetyl, the final product in the oxidation. See Butanediol above.

The methods are described by Kniphorst and Kruisheer (Food Experiment Station, Enshede, Holland) in the reverse order, since the determination of diacetyl as the nickel compound (nickel dimethylglyoxime), $Ni[(CH_3)_2(CNO)_2H]_2$, is the basic procedure.

PROCESS. A. DIACETYL (Modified Lemoigne-van Niel Method). *Distillation.* Pipet into a 250-ml. fractioning flask 25 ml. of the slightly acid sample containing 5 to 50 mg. of diacetyl and add 25 ml. of water. Close the flask with a rubber stopper, the lower surface of which is covered with Durofix, an inert membranous material. Connect the flask with a condenser and the latter with a delivery tube that dips below the reagent prepared by mixing with 25 ml. of water, 6 ml. of *10% nickel chloride solution*, 2 ml. of *20% hydroxylamine hydrochloride solution*, and 5 ml. of *20% ammonium chloride solution*, contained in an ice-cold 200-ml. Erlenmeyer flask. Distil so as to collect 30 ml. of distillate in about 30 minutes. If acetyl-methyl carbinol is present, the distillation must be conducted in a stream of *carbon dioxide;* otherwise it is oxidized to diacetyl. Add to the distillate dropwise with shaking 0.5 ml. of *20% ammonium hydroxide.*

If more than 10 mg. of diacetyl is present, the color of the solution changes to yellow and a red precipitate soon separates; if less than 5 ml. is present (this is usually indicated after adding 0.3 ml. of 20% ammonium hydroxide by the absence of a color change to yellow-green), add no more of the alkali.

As directed by Schmalfuss and Rethorn, place the flask in a bath of cold water, heat to boiling, and continue the boiling 1.5 hours, then cool and keep in an ice bath for 1 hour. Collect the precipitate in a crucible with a perforated porcelain bottom (Schott & Gen. 1 G 3) and wash with 100 ml. of ice-cold water. Dry for 30 minutes in an oven at 110°, cool for 30 minutes in a desiccator, and weigh.

CALCULATION. To obtain the weight of diacetyl, multiply the weight of the nickel dimethylglyoxime by 0.596.

B. ACETYL-METHYL CARBINOL. Oxidation to diacetyl by ferrous sulfate in addition to ferric chloride, in the present modification, produced a yield of 97% as compared with that of the van Niel modification, using only ferric chloride, which is only 91%.

In the absence of diacetyl, place 25 ml. of the solution, containing not more than 50 ml. of acetyl-methyl carbinol, in a 250-ml. fractioning flask. Add 5 g. of *pulverized crystallized ferrous sulfate* and 25 ml. of *30% ferric chloride solution*, then heat for 15 to 20 minutes with a small flame, gradually attaining

the boiling point; after this distil the diacetyl formed and proceed in other details exactly as described for the determination of diacetyl.

C. 2,3-Butylene Glycol (Modified Lemoigne Bromine-Ferric Chloride Method). The oxidation to acetyl-methyl carbinol by bromine is followed by further oxidation to diacetyl as given above.

If only butylene glycol is present, place 25 ml. of the solution (in the case of wine, 17 to 18 ml. of distillate from 25 ml.), containing not more than 50 mg. of that substance, in a 100-ml. Erlenmeyer flask, add 0.25 ml. of *bromine*, and shake until the bromine has nearly all dissolved. Connect the flask with a reflux condenser, immerse in a water bath at 70 to 80°, shake for about 30 seconds, keep at that temperature in the water bath for exactly 20 minutes additional, then replace the hot water in the bath with cold and add some cold water to the mixture in the flask. To combine with the excess of bromine, add about 2.5 g. of *pulverized ferrous sulfate* until the color and odor of the bromine disappear, then add 5 g. of *ferrous sulfate* additional and 20 ml. of *30% ferric chloride solution.* Determine the acetyl-methyl carbinol thus formed as described above under B.

Calculation. Nickel dimethylglyoxime $\times$ 0.624 = butylene glycol.

Reducing Sugars

Copper Reduction Gravimetric Method. Neutralize 50 to 200 ml. (depending on the content of extract) with *dilute sodium hydroxide solution*, dealcoholize by evaporation to from one-third to one-half of the original volume, and remove to a 200-ml. volumetric flask. Clarify with *1.0 N lead acetate solution*, make up to the mark with water, shake, and filter through a dry paper. Delead with dry *potassium oxalate*, filter, and determine the invert sugar (reducing sugars) by the Munson and Walker Method, Part I, C6*a*. Calculate as grams per 100 ml.

Sucrose

Copper Reduction Method. Invert an aliquot of the solution obtained in the determination of reducing sugars, as described above, neutralize, and determine the invert sugar by the Munson and Walker copper reduction method. Multiply the difference between the invert sugar before and after inversion by 0.95 to obtain the weight of sucrose.

If preferred, polarize before and after inversion and calculate the sucrose by formula.

Tannin

Neubauer-Löwenthal Permanganate Volumetric Method.[55] This method or the Schroeder modification [56] is a standard method in Germany, Austria, and the United States.

Reagents. *Oxalic Acid Solution*, 0.1 *N*. Dissolve 6.3 g. of crystalline oxalic acid in water and make up to 1 liter at 20°; 1 ml. = 0.004157% of tannin.

Standard Potassium Permanganate Solution. Add 1.333 g. of $KMnO_4$ to 1 liter of water and heat until dissolved. Standardize against the 0.1 *N* oxalic acid.

Indigo Solution. Add 6 g. of sodium indigotin-sulfonate to 500 ml. of water and heat until dissolved.

Bone Black. Purify by boiling 100 g. of finely powdered bone black with 10% HCl, wash with boiling water until neutral, and store covered with water in a bottle.

Process. Evaporate 100 ml. of the accurately measured sample to one-third to one-quarter of its original volume, to remove the ethanol, and dilute to the mark in a 100-ml. volumetric flask. Pipet 10 ml. into a 2-liter porcelain dish, dilute with water to 1 liter, and add 20 ml. of *indigo solution* from a pipet. Titrate with *standard permanganate solution*, adding 1 ml. at a time until the blue color changes to green, then reduce the rate to a

few drops at a time until the color changes to golden yellow.

Shake another portion of 10 ml. of the dealcoholized sample with *bone black* for 15 minutes, filter, and wash thoroughly with cold water. To the filtrate add 1 liter of water and 20 ml. of *indigo solution*, and titrate with *permanganate solution* as before.

Calculation. To obtain the grams of tannin (plus accompanying color) in 100 ml. of the wine, multiply the difference in the number of milliliters of the permanganate solution required for the two titrations by 10 and the product by the weight of tannin equivalent to 1 ml. of the permanganate solution.

Schroeder Modification. This differs from the original method chiefly in that *hide powder*, washed free of soluble organic matter, is substituted for bone black and the standard of the permanganate solution (1.667 g. per liter) is fixed by titration against 0.2% tannin solution.

Nitrates

Egger Diphenylamine Test.[57] Add a few drops of the sample to 2 to 3 ml. of a solution of 0.1 g. *diphenylamine* in 100 ml. of *sulfuric acid*. The blue color indicative of nitrates forms quickly before the sugar of sweet wine is charred sufficiently to obscure the reaction. Before making the test, clarify colored wines with *basic lead acetate*, filter, delead the filtrate with *sodium sulfate*, filter again, and further clarify with *bone black* if necessary.

Ash

Incineration Method. Evaporate 25 ml. of the sample in a flat-bottom platinum, quartz, or porcelain dish, burn below redness to a white ash, and determine the total, water-soluble, and water-insoluble ash, also the alkalinity of each as described in Part I, C2*f*.

Sodium Chloride

Volhard Silver Chloride Volumetric Method. Evaporate 50 or 100 ml. of the wine with an excess of *sodium carbonate*, ignite below redness, dissolve in *water acidified with nitric acid*, filter, wash, and determine chlorine as silver chloride. See Part I, C8*a*.

Potassium Sulfate

Plastered wine is made from must to which calcium sulfate has been added, the reaction being as follows:

$$+ CaSO_4 -$$

$$CaC_4H_4O_6 + \quad\quad +$$

The calcium tartrate formed is precipitated, thus reducing the content of tartaric acid, but the potassium sulfate is correspondingly increased.

In several European countries plastering is permitted provided the potassium sulfate does not exceed certain limits differing with the kind of wine.

Barium Sulfate Gravimetric Method. To 50 ml. of the sample, add a few drops of *hydrochloric acid*, heat to boiling, and precipitate the sulfuric acid with *barium chloride solution*. After allowing to stand overnight in a warm place, collect the barium sulfate on a filter, wash, ignite, and weigh.

Calculate as grams of potassium sulfate per 100 ml.:

$$BaSO_4 \times 0.7465 = K_2SO_4$$

Bromine

Bromine, added to wine and other foods in the form of bromoacetic acid, may be detected and determined as follows.

Florentin and Munsch Magnesium Oxide Test.[58] Extract 20 to 200 ml. of the wine or other liquid under examination with 50 to 250 ml. of *ether*. Evaporate the ether solution in

a dish containing 1 to 2 ml. of water designed to dissolve bromoacetic acid, thus preventing its loss by volatilization. After the ether has been driven off, add a little *bromine-free magnesium oxide,* heat with a low flame, and finally increase the heat to a temperature sufficient for gentle ignition. Take up the residue in a little boiling water and apply the Denigès and Chelle or Hahn Test (Part II, A2), the former being accurate to 0.5 mg., the latter to 0.1 mg. of bromine.

Fluorine

Willard and Winter Thorium Nitrate Method. See Part I, C8*b*.

Rempel Modification.[59] In the following modification, developed at the Twining Laboratories, Fresno, California, the Willard and Winter procedure has been simplified and adapted to the detection of fluorine spray residues in the wine industry.

Process. *Incineration.* Pipet 50 ml. of the sample into a platinum dish, neutralize with *saturated sodium carbonate solution,* evaporate to near dryness on a water bath, then complete the drying by directing a moving free flame from above and burn in an electric muffle at 525°.

Solution. Take up in 10 ml. of water, transfer to a 125-ml. distilling flask, using no more than 25 ml. of water.

Distillation. Add 12 ml. of *60% perchloric acid* and steam-distil in an oil bath heated at 150°,[60] keeping the liquid at 130 to 140°, as measured by a thermometer reaching to within 0.5 cm. of the bottom of the flask, and regulating the rate so as to collect 200 to 250 ml. in 45 to 60 minutes.

Adjustment of Reagent. Add to the distillate *saturated sodium carbonate solution* until alkaline to *phenolphthalein,* then evaporate below 85° to about 10 ml., cool, and add *1 + 20 hydrochloric acid* sufficient just to discharge the pink color, followed by 1 ml. of *0.05% sodium alizarin sulfonate solution*[61] to replace the zirconium nitrate and alizarin red mixture used by Willard and Winter. Discharge the red color by adding *0.01 N hydrochloric acid* dropwise to an acidity of 5.0 *p*H. Dilute the adjusted solution to the mark in a 100-ml. flask with water at *p*H 5 and divide into two parts (for duplicate determinations), adding to each 50 ml. of *ethanol,* also at *p*H 5.

Titration. Add at first 0.1 ml. of *standard 0.01 N thorium nitrate solution* (the amount required by the indicator). If no end-point is reached with this amount (indicating the presence of fluorine), complete the titration slowly, stirring vigorously and allowing to stand at least 15 seconds after each drop.

Calculation. The volume of *standard thorium nitrate solution,* above 0.1 ml. represents the fluorine in the sample. The value of each milliliter is determined by standardization against *standard sodium fluoride solution.*

Iron

Blau Phenanthroline Volumetric Method.[62] Blau obtained an intense red ferrous-*o*-phenanthroline complex which Walden, Hammett, and Chapman[63] found may be used as a reversible oxidation indicator of high potential and is well suited for many oxidimetric titrations.

Saywell and Cunningham Colorimetric Modification.[64] The modification was developed at the College of Agriculture of the University of California.

Process. *Wet Combustion.* Digest in a Pyrex test tube over a free flame with agitation 2 ml. of fruit juice or wine with 1 ml. of *sulfuric acid* and 0.7 to 1.0 ml. of *60 to 70% perchloric acid.* If the combustion is not complete in 2 to 10 minutes, use 0.5 to 1 ml. additional *perchloric acid* and continue the heating until the solution is water-white.

Color Formation. Add 1 ml. of water, followed by 1 ml. of *10% hydroxylamine hydrochloride solution* with shaking and 0.5 ml. of *1.5% o-phenanthroline solution,* then *ammonium hydroxide* (about 2 ml.) until a light red or pink color appears. Drop a small piece of

Congo red paper into the solution and continue the addition of *ammonium hydroxide* until the paper is distinctly pink.

Color Comparison. Dilute the solution to the 10- or 15-ml. graduation mark and mix thoroughly. Match the solution against *standard iron solutions* containing 20, 15, 10, and 5 γ/g. (or a series with steps of 2.5 γ/g.) treated in like manner.

EXAMPLES. Average of results by the phenanthroline, Lyons thioglycolic acid, and Stugart thiocyanate methods are respectively as follows: Muscatel 3.5, 3.3, and 3.2, Sherry 3.3, 2.9, and 3.3, Port 3.2, 3.2, and 3.4, Sauterne 5.3, 5.2, and 4.9, Riesling 7.4, 7.5, and 7.6, and Claret 4.0, 3.9, and 3.9 γ/g.

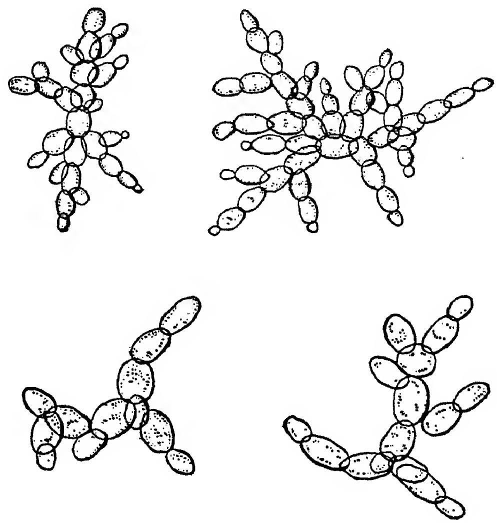
2. MALT LIQUORS

In the manufacture of malt liquors the conversion of starch in the wort into the soluble carbohydrate maltose is first effected by means of *diastase*, the enzyme of malt, then the maltose is hydrolyzed by means of the enzyme *maltase* (maltoglucase), with the formation of dextrose as follows:

$$\underset{\text{Starch}}{} \qquad \underset{\text{Maltose}}{C_{12}H_{22}O_{11}}$$

$$\underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + H_2O \cdot \quad \cdot \underset{\text{Dextrose}}{2\ C_6H_{12}O_6}$$

For the fermentation of malt liquors, yeast of the species *Saccharomyces cerevisiae* is added. In making lager beer, a strain known as bottom yeast (Fig. 148, II) is used, whereas for ale, top yeast (Fig. 148, I) is necessary. Malt liquors may be made exclusively from malt, but in the United States other cereal products, notably unmalted barley, corn grits, and rice, are also used.



II

FIG. 148. I, Top, Ale, or Distillery Yeast; II, Bottom or Beer Yeast. Budding Plants. (Lindner.)

Chemical Composition. The average composition of malt liquors (results other than ethanol in terms of grams per 100 ml.), compiled from analyses reported by König,[65] follows:

	Ethanol by Weight	Extract	Acid as Lactic	Glycerol	Ash	Phosphoric Acid	Nitrogenous Substances	Sugar as Maltose
Lager beer	3.93	5.79	0.15	0.17	0.23	0.077	0.71	0.88
Bock beer	4.69	7.21	0.17	0.18	0.26	0.089	0.73	1.81
Ale	4.75	5.65	0.28		0.31	0.086	0.61	1.07
Porter	4.70	6.59	0.28		0.36	0.093	0.65	2.62

Other constituents determined in malt liquors are: dextrin, protein, added bitter principles and preservatives, and arsenic introduced in glucose made with impure acid.

Sample

Transfer the contents of a bottle or an equal volume from the keg, both at room temperature, to a large flask, shake gently at first, then vigorously, and filter through a dry paper to remove froth or suspended matter.

Most of the methods which follow are essentially those of the Association of Official Agricultural Chemists (A.O.A.C.) and of the American Society of Brewing Chemists (A.S.B.C.).

Color

Filter if not clear. Determine the color value in a half-inch cell of the Lovibond tintometer, using series 52 slide and standard daylight lamp (A.O.A.C.).

Specific Gravity

Determine at 20°/20°. Special precautions are given in the *A.O.A.C. Methods of Analysis*. If desired, calculate the specific gravity *in vacuo* (G) by the formula

$$G \quad \frac{S + 0.00105W}{W + 0.00105W}$$

in which S and W are the weights at the desired temperature in air of the contained sample and contained water respectively.

Extract

(Real Extract)

All the methods below have been adopted by the A.O.A.C. and all but A by the A.S.B.C. Domke's table (Part II, E2) may be used in place of Plato's table.

A. Dealcoholization Method. Use the Dealcoholization Method given under Wine (above).

B. Evaporation Method. Accurately measure and weigh 100 ml. of the sample. Evaporate on a water bath at 80° or below to one-third the original volume. Cool, add water to the original weight and determine the specific gravity at 20°/20°. Obtain the real extract (sucrose) from Plato's table (*Official and Tentative Methods, A.O.A.C.*).

C. Distillation Method. If no anti-foam was used in the determination of ethanol, transfer the residue with hot water to a 100-ml. pycnometer, cool to 20°, make up to the mark, and weigh. The weight divided by 100 is the specific gravity. Find the per cent of sucrose (extract) corresponding to the specific gravity directly in the Plato table. If 100 ml. of beer were used, obtain the grams of extract per 100 g. of beer (G) by the formula

in which E is the extract found and D and B are the specific gravities of the dealcoholized beer and the original beer respectively.

Extract of Original Wort

Beythien Calculation Method.[66] Although much used, the following formula has been criticized by several authors:

$$W : \quad \frac{+ 2.0665A)}{100 + 1.0665A}$$

in which W is the percentage of extract in the original wort, E is the percentage of real extract in the beer, and A is the percentage of ethanol by weight in the beer.

The two American associations give the same formula.

For most practical purposes, the following simpler formula is deemed adequate:

$$W = E + 2A$$

Apparent Extract

Find in the Domke table (Part II, E2) the percentage of apparent extract (sucrose) corresponding to the specific gravity 20°/20° and report to the second decimal place (A.O.A.C., A.S.B.C.).

Real Degree of Fermentation

Calculation Method. Obtain the rèal extract degree of fermentation (D) by the formula

in which W is the original extract and E is the real extract (A.O.A.C., A.S.B.C.).

Extract and Ethanol

Berglund, Emlington, and Rasmussen Calculation Method.[67] The formulas, based on Goldiner and Kemann extract tables and the Block alcohol table, both at 20°/20° gave results with the mean deviation of ± 0.03 for the extract and ± 0.04 for ethanol on 52 samples from 17 Scandinavian breweries.

$$E = 0.251 + 1.298S + 0.1179R$$

$$A = 0.323 - 2.774S + 0.2691R$$

in which E and A are grams of extract and ethanol respectively in 100 g. of beer, S is the specific gravity less 1 multiplied by 100, and R is the difference between the refractometer scale readings for the degassed beer and for water, both at 20°.

Protein

Kjeldahl Method. Pipet 50 ml. of the sample into a Kjeldahl flask, add 5 ml. of *sulfuric acid*, evaporate to a sirup, then add 15 ml. more of the *acid*, and proceed as directed for the Kjeldahl-Willfarth or Kjeldahl-Gunning method in Part I, C1*c*. (A.O.A.C., A.S.B.C.).

Total Acids

Volumetric Method. Pipet 10 or 25 ml. of decarbonated beer into 10 volumes of boiling water and boil 60 seconds or longer. Cool to room temperature and titrate at once with *0.1 N alkali*, using 0.5 ml. of *0.5% phenolphthalein* as indicator; 1 ml. of *0.1 N* alkali = 0.0090 g. of lactic acid (A.O.A.C., A.S.B.C.).

Note. Luck[68] uses as outside indicator a mixture of 20 ml. of boiled water, 10 to 12 drops of *3% ethanolic phenolphthalein indicator*, and 0.2 ml. of *0.1 N sodium hydroxide solution*. Neutral litmus paper gives lower results.

Volatile Acids

See Wine above; 1 ml. of 0.1 N alkali = 0.006 g. of acetic acid (A.O.A.C., A.S.B.C.).

Reducing Sugars

Copper Reduction Methods. Determine by the Lane and Eynon or the Munson and Walker method (Part I, C6*a*). Calculate as grams of maltose per 100 ml. of beer (A.O.A.C., A.S.B.C.).

Dextrin

Acid Hydrolysis-Copper Reduction Method. Pipet 50 ml. of the decarbonated sample at 20° into a 200-ml. volumetric flask, add 15 ml. of *hydrochloric acid* (sp.gr. 1.125), make up to the mark, and hydrolyze by refluxing in a boiling water bath for 2 hours. After cooling, nearly neutralize with *sodium hydroxide solution*, make up to 250 ml., filter, and determine the copper reduction by the method used for the determination of reducing sugar.

From the weight of copper, derive the weight of dextrose equivalent to 100 ml. of the sample, subtract from that weight 1.053 times the grams of maltose, and multiply the

remainder by 0.9. The product is the grams of dextrin per 100 ml. of the sample (Tentative A.O.A.C. Method).

Ethanol

Distillation Method. Proceed as directed for Wine above, omitting, however, the neutralization with sodium hydroxide solution. Add *tannin* or other anti-foam only when necessary (A.O.A.C., A.S.B.C.).

Immersion Refractometer Method. Determine the refraction of the distillate at 17.5° and find the equivalent percentage of ethanol by volume in the table. See Distilled Liquors below (A.O.A.C.).

Methanol

See Distilled Liquors below (A.O.A.C.).

Glycerol

Usually beer contains less than 0.20% of glycerol or only about one-fifth that in wine.

Determine by one of the methods described in Part II, F1 and D3 (A.O.A.C.).

Carbon Dioxide

Since subsampling of beer without loss of gas is impracticable, determination of carbon dioxide is carried out on the entire contents of a bottle. To do this with all the beer in a keg is possible but it would require absorption apparatus as large as that of the respiration calorimeter.

Three methods are described: (1) the direct method, weighing the carbon dioxide in a potash bulb or soda lime tube, which is accurate but laborious, (2) the indirect method, weighing the bottle and contents before and after removal of the gas, which is sufficiently exact for some purposes and less exacting, and (3) the manometric method.

Direct Gravimetric Method. Apparatus. That devised for baking powder (Part II, K2), modified so as to deliver the gas from the bottle to the condenser, is well suited for the determination.

If the closure is by a cork, which now is unusual, a champagne tap with a cock meets the requirements, since it bores without loss of gas. When the aspiration is well started, the cork with tap is replaced by a double-bored rubber stopper, through one hole of which a tube connected with a soda lime tube is passed to the bottom of the bottle and through the other a short outlet tube is connected with the condenser of the train.

The crown cap, which has almost entirely replaced the cork, presents greater difficulties which, however, may be met either (1) by removing the cap and quickly inserting in its place a double-bored rubber stopper, such as is noted above, or, better, (2) by clamping or wiring a single-bored stopper against the cap and passing a special tap with an auger end through the greased hole of the stopper and then boring through the cap without loss of gas.

Process. The process is so conducted as to avoid too rapid evolution of gas, the entry of carbon dioxide with the air during aspiration or, what is more disastrous, the loss of carbon dioxide by back pressure. Follow in other details the direction given for baking powder.

The maximum carbon dioxide content in the beer in numerous determinations reported by König [69] is well within 0.50% and the average content is within 0.25%, hence the weight of gas in the standard 12-ounce bottle, containing about 350 ml., is less than 2 g. and usually less than 1 g. The latter figure is not far from 4 times that in the usual charge of 2 g. of baking powder containing 14% of carbon dioxide. This larger amount necessitates care in regulating the evolution of gas and more frequent renewal of the soda lime by which it is quantitatively absorbed.

Bode Indirect Gravimetric Method.[70] APPARATUS. *Balance* with a capacity of 1 kg. and sensitive to 10 mg.

Triple Bulb Tube carried by a single-bored rubber stopper fitted to the mouth of a beer bottle. The form designed for the Varrentrapp and Will nitrogen combustion, but with the narrow entrance portion bent downward to a vertical position, is suitable.

PROCESS. Partly fill the bulb tube with *sulfuric acid,* allowing room for the passage of air and carbon dioxide, and introduce a piece of *tallow* or *paraffin* the size of a pea. Cool the stoppered beer bottle, wipe dry, and weigh together with the contents, cork or cap, bulb tube, rubber stopper, and 3 pieces of *pumice stone.* Open the bottle, reserving the cork or cap for the final weighing, introduce the *pumice stone,* attach the bulb tube, and place in a water bath.

Heat the bath gradually and when the evolution of gas slackens bring to boiling and boil 15 minutes. Attach a calcium chloride tube to the bulb tube to prevent entrance of moisture from without and allow to cool. Dry the bottle and weigh with accessories as before, obtaining the carbon dioxide by difference.

Empty the bottle, wash, rinse with *ethanol* and *ether*, dry, weigh, and determine the weight of the original beer by difference.

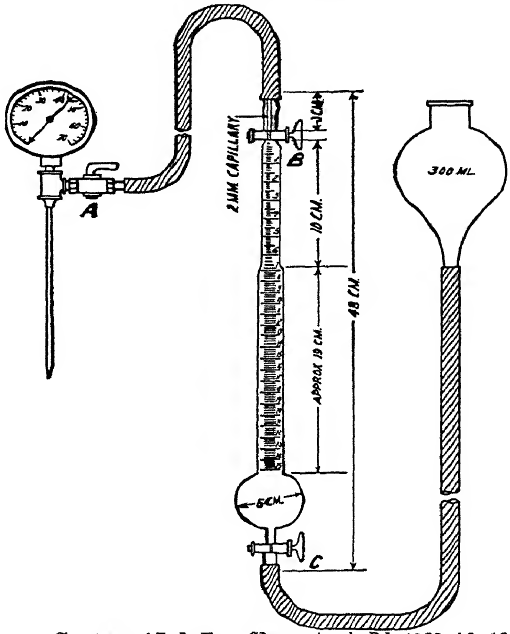
CALCULATION. Calculate the percentage of carbon dioxide, adding 0.04% to correct for unavoidable loss in the operation.

NOTE. More complete removal of the gas can be secured by aspiration, but this involves using a double-bored stopper with an entrance tube reaching into the liquid and connected with soda lime and calcium chloride tubes to avoid entrance of carbon dioxide or moisture.

Gray and Stone Manometric Method.[71] The method (Wallerstein Laboratories, New York) is suited for packaged beer and other carbonated beverages.

APPARATUS (Fig. 149). *Piercing Device,* with hollow spike connected with a pressure gage, used in connection with a packing box for bottles and a metal frame for cans.

Absorption Buret, consisting of (1) a tube graduated from 0 to 6 ml. in 0.1-ml. divisions, joined to (2) a tube graduated from 6



Courtesy of Ind. Eng. Chem., Anal. Ed. 1938, **10,** *16*

FIG. 149. Gray and Stone Piercing and Manometric Apparatus.

to 25 ml. in 0.2-ml. divisions, and this in turn to (3) a bulb 5 cm. in diameter, the whole provided with stopcocks at each end and a leveling bulb.

PROCESS. *Puncturing Cap or Can.* Make a scratch at the level of the beer in a bottle; weigh a can and contents. Submerge in a water bath at 25° long enough to cool the beer to that temperature. Place in the piercing apparatus and fill the bulb and buret with *15% sodium hydroxide solution* up to cock *B*. Fill the upper capillary with

hexyl alcohol and the remainder of the tube to the tip of the spike with water. With outlet cock A closed, drive the spike through the crown or top of can, shake well, and tap the container.

Pressure Reading. Read the pressure gauge, then shake and take pressure readings, using the one that remains fixed in consecutive readings.

Open stopcocks B and C, then stopcock A and allow the gas together with foam to flow over into the absorption bulb. Swirl the contents in the bulb to hasten the absorption of the carbon dioxide. When half to three-fourths of the liquid in the bulb has been displaced, shut all stopcocks and shake. Set the bulb in a vertical position, open C, and allow the alkali to flow back into the bulb. Open B and A and repeat the operation while tapping the container. Close A and B and again shake to absorb the last traces of gas.

Air Reading. Read the volume of unabsorbed gas (air) with the liquid in the buret and leveling bulb in the same plane. Repeat until constant.

Head Space Determination. Disconnect the container. If a bottle, fill with water to the top and pour off into a graduated cylinder to the scratch mark. Record the number of milliliters of head space. If a can, pour out all remaining beer and weigh the empty can. Subtract from the former weight, thus obtaining the weight of beer which, divided by the specific gravity of the beer, gives the volume of beer in milliliters. Fill the empty can with water and weigh. The weight of water in grams is also the volume in milliliters, hence the difference between the volume of water and that of beer represents the head space.

CALCULATION. Obtain the percentage of carbon dioxide by weight (C) by the formula

$$C = \quad - \left(\frac{A}{H}\right) \times 14.7 \Big] \times 0.00965$$

in which P is the absolute pressure in pounds per square inch at 25°, A is the milliliters of air, and H is the milliliters of head space.

For ordinary work, use 15 instead of 14.7.

The *Methods of the American Society of Brewing Chemists* give also the following formulas for calculating the volume of CO_2 (V):

$$V = \frac{C}{0.1976} \times S \quad \text{or} \quad V = C \times 5.0607 \times S$$

in which C is the per cent of carbon dioxide by weight and S is the specific gravity of the beer.

Ash

Evaporate and ignite below redness in a platinum, quartz, or porcelain dish 50 ml. of the decarbonated sample or ignite the residue after determination of the extract by the direct method and weigh.

Calculate the result as grams per 100 ml. (A.O.A.C., A.S.B.C.).

Phosphoric Acid

Molybdate Gravimetric Method. Evaporate and ignite 50 ml. of the decarbonated sample together with a few drops of *concentrated magnesium nitrate solution.* Proceed as directed in Part I, C8*a*. (A.O.A.C., A.S.B.C.)

Calculate the result as grams of phosphoric acid (P_2O_5) per 100 ml.

Sulfuric Acid

Barium Sulfate Gravimetric Method. Evaporate to dryness, on an electric stove or over an alcohol flame, 50 ml. of the decarbonated sample, mix with 1 g. of *sodium carbonate* and a few drops of *10% sodium nitrate solution,* and ignite below redness. One gram of *sodium peroxide,* added in successive portions toward the end of the evaporation, may be substituted for the sodium nitrate solution, in which case the ignition must be conducted in a nickel crucible. To the residue, add 10 ml. of water and 1 ml. of *hydrochloric acid,* evaporate nearly to dryness to

remove the excess of acid, dilute to 50 ml., boil, and add *barium chloride solution.* After 3 hours, collect the barium sulfate on a filter, wash, ignite, and calculate as grams of sulfuric acid (SO_3) per 100 ml.

Iron

Hill Dipyridyl Colorimetric Method.[72] Hill proposed the method and applied it to various biological materials.

Gray and Stone Modification.[73] As used in the Wallerstein Laboratories, New York, the procedure is as follows.

Apparatus. *Simple Block Comparator.*

Reagents. *Bipyridine Reagent.* To 100 mg. of 2,2′-bipyridine add 2 ml. of 1 + 2 acetic acid. Mix and dilute to 50 ml. with water.

Standard Iron Solution. Dissolve 3.512 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Mohr's salt) in water, add 2 drops of HCl, and dilute to 500 ml. with water. Dilute 10 ml. of this solution to 1 liter; 1 ml. = 0.01 mg. of iron; 1 ml. of this solution diluted to 10 ml. = 1 γ/g.

Permanent Standards. Prepare in test tubes solutions containing 0 to 0.05 mg. of iron in 0.005-mg. steps, add to each 0.5 ml. of $Na_2S_2O_4$ solution (freshly prepared 2%), and dilute to 10 ml., then add 0.5 ml. of the bipyridine reagent and mix. Cork the tubes and seal with paraffin. These standards will keep for months.

Process. *Color Formation.* Place 10 ml. of the degassed sample in each of three test tubes; to one add 0.5 ml. of the *bipyridine reagent* and mix. Heat in a water bath at 70° for 30 minutes and cool.

Color Comparison. Arrange the tubes containing the unknown and permanent standards in a comparator block and match the colors.

Chlorine

Silver Nitrate Methods. Evaporate 50 ml. of the sample with 1 g. of *sodium carbonate*, ignite at dull redness, take up in boiling water, and determine chlorine gravimetrically or volumetrically as silver chloride. See Part I, C8*a*. (A.O.A.C.)

Tin

Aside from being objectionable from the hygienic viewpoint, as little as 0.1 γ/g. of tin produces a haziness in malt liquors.

Stone Mercaptobenzene Nephelometric Method.[74] The method was developed at the Wallerstein Laboratories, New York.

Apparatus. *Comparison Tubes.*

Reagents. *Dithiol Reagent.* Dissolve 0.25 ml. of dithiol (1-methyl-3,4-dimercaptobenzene), previously liquefied by gentle heating, in 10 ml. of thioglycolic acid, and dilute with ethanol to 200 ml. Store in tightly closed bottles in a dark place. Reject when tests show a diminution in strength.

Fusion Mixture. Grind quickly (to avoid evolution of hydrocyanic acid by atmospheric carbon dioxide) to a homogeneous powder in a mortar 12.5 g. of NaCN with 37.5 g. of Na_2CO_3.

Standard Tin Solution. Dissolve 1.90 g. of stannous chloride dihydrate in 20 ml. of 1 + 1 HCl and dilute to 1 liter with water. The solution should be freshly prepared. Dilute 20 ml. of this stock solution and 10 ml. of HCl to 200 ml. with water and add to six test tubes 0.0, 0.1, 0.25, 0.50, 0.75, and 1.0 ml. equivalent respectively to 0.0, 0.01, 0.025, 0.05, 0.075, and 0.1 mg. of tin. Add to each 1 ml. of 1 + 1 HCl and dilute to exactly 10 ml. with water. Add 0.5 ml. of dithiol reagent, mix, and heat in a slowly boiling water bath for 1 minute. Cool, add 2 ml. of gum arabic solution, cork, and shake well. These solutions are stable for at least a month, but should be shaken before using.

Gum Arabic Solution. Dissolve 100 g. of powdered gum arabic in 1 liter of hot water containing 100 ml. of 0.1% phenyl mercuric acetate solution. Filter through paper in a

hot funnel. The solution is stable and will not get moldy.

PROCESS. *Incineration.* Char and ignite 100 to 200 ml. of the degassed sample in a silica dish, avoiding fusion. Brush the fluffy ash into a No. 00 Coors high-form porcelain crucible, tamp, and cover with 1 g. of the *fusion mixture.*

Fusion. Fuse cautiously for about 15 seconds over a Méker burner, holding the crucible in tongs and mixing by a swirling motion. Cool, place the crucible in an upright position in a 50-ml. Pyrex beaker, cover the beaker with a watch glass, place in a well-ventilated hood, and guard against breathing the highly poisonous gas in the subsequent operations.

Solution of Tin Oxide. Pipet 5 ml. of *1 + 1 hydrochloric acid* directly into the crucible through the lip of the beaker. When the evolution of gas ceases, remove the watch glass and rinse with water into the crucible, then overturn the crucible and heat, thus causing a further evolution of the highly toxic gas. Continue the heating and stirring until the evolution of gas ceases, then remove the crucible with a rod, rinse with water, and again cover the beaker. Concentrate the solution by boiling to less than 10 ml. Transfer the solution to a 15-ml. centrifuge tube with a mark showing 10 ml., make up to that volume, mix, and centrifuge at high speed. Transfer the clear solution to a test tube, add 0.5 ml. of the *dithiol reagent,* mix, and heat for 1 minute in a gently boiling water bath. After cooling, add 2 ml. of the *gum arabic solution,* cork, and shake well.

Turbidity Comparison. Make the comparison by reflected daylight with well-shaken *standard tin solutions.*

3. DISTILLED LIQUORS

Classified according to origin, certain distilled liquors, such as whiskey, American gin, and cordials made from grain, belong under Cereal Products, others, such as cognac, cider brandy, and other fruit brandies, under Fruit Products, and others still, such as rum and rum cordials, under Saccharine Products. Potable liquor, made on a large scale in Germany from potatoes, may be classified under Vegetable Products.

Chemical Composition. The composition of distilled liquors, compiled from various sources, is shown in the table below.

	Analyst	Ethanol % by Volume	Grams per 100 Liters of Proof Spirits						
			Extract	Acids		Esters	Alde-hyde	Fur-fural	Fusel oil
				Total	Vola-tile				
Whiskey									
Scotch, 8 years old	Vasey	....	.....	....	24.0	44.8	7.1	2.0	100.0
Irish, 7 years old	Vasey	....	.....	....	20.9	10.5	5.6	1.7	102.0
Rye, 4 years old	Crampton and Tolman	55.6	185.0	65.9	....	69.3	13.9	2.8	125.1
Bourbon, 4 years old	Crampton and Tolman	52.2	151.9	58.4	....	53.5	11.0	1.9	123.0
Imitation rye	Ladd	45.0	506.1*	10.6	7.3	5.7	trace	0.9	46.9
Cognac, 10 years old	Vasey	....	.....	....	37.2	54.6	8.3	0.9	62.1
Rum	Vasey	....	.....	....	14.0	199.5	4.2	1.4	45.3
Gin	Vasey	....	.....	....	0.0	18.7	0.9	0.0	22.3
Neutral spirits	Ladd	94.0	1.2	3.8	3.8	14.0	3.2	trace	14.8

* Includes caramel color.

Tests are also made for methanol, caramel, and other artificial colors, and artificial flavors.

Sample

The liquor for analysis must be perfectly clear. If there is a sediment, decant from it; if turbid, filter. The complication of making a decarbonated and dealcoholized preparation, which is essential in the analysis of effervescent malt liquors, is obviated, since distilled liquors are never effervescent and do not contain solids that change materially during drying at the temperature of boiling water.

Specific Gravity

Calibrate the pycnometer or hydrometer at 20° and make all determinations on the liquor and on the distillate in ethanol determination at that temperature. The gauger's hydrometer is an instrument which in the analysis of most distilled liquors, like the saccharimeter in the analysis of refined sugar, furnishes remarkably accurate results, since in distillation only two products, ethanol and water, pass over in substantial amounts, and hydrometers are graduated and alcohol tables are calculated on this basis.

Extract

(Solids)

Evaporate to dryness 50 g. or 50 ml. of the sample in a tared flat-bottom platinum, quartz, or porcelain dish on the steam bath. Complete the drying in a boiling water oven for 2.5 hours, cool, and weigh.

Calculate as percentage by weight or grams per 100 ml.

Total Alcohols

Aiyar and Krishnan Modification of Thorpe and Holmes Method.[75] In taxing liquors in India, ethanol, methanol, propanol, and isopropanol are grouped as alcohol.

Process. When isopropanol is suspected, saturate 100 ml. of liquid (original sample or distillate), containing 30% of proof spirit, with *sodium chloride*, shake with 50 ml. of *naphtha* (b.p. 40–60°) and allow to settle. Drain off the brine and wash the naphtha extract with 10 ml. of *saturated sodium chloride solution* and five times with 10-ml. portions of water. Combine the brine and aqueous washings, remove 100 ml. by distillation, and determine its specific gravity.

Ethanol

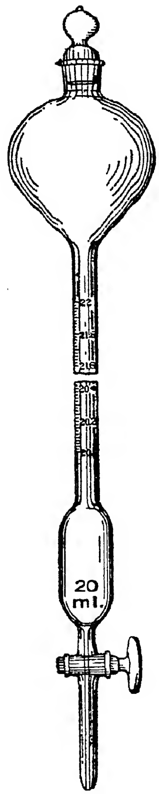
No little confusion is caused by linking weight to volume (grams per 100 ml.) and by the dual system of ethanol by weight and by volume. So-called 95% alcohol has approximately that percentage of ethanol by volume, but the corresponding percentage by weight is approximately 2.5% less. In proof liquors (50% by volume in the United States), the difference is about 1.6%. A distinct advantage of the volume system is that dilution to any percentage can be made by the nonchemist without recourse to tables. In general food analysis, however, the tendency is away from percentage by volume and grams per 100 ml. and toward percentage by weight.

Distillation Method. Process. Deliver into a tared 250-ml. distilling flask from an accurate pipet 25 ml. of the liquor and weigh together with the flask. (If only percentage by weight is desired, employ exactly 25 g. to simplify the calculation.) Dilute with 125 ml. of water and connect with the upright condenser of the assembly shown in Fig. 145 under Wine above. Attach to the lower end of the condenser the adaptor previously introduced into the weighed pycnometer. Distil nearly 100 ml. as described under Wine, cool the distillate to 20°, make up to the mark at 20°, and weigh.

CALCULATION. To obtain the percentage by weight or by volume, follow the directions given under Wine above and multiply the result by 4.

FUSEL OIL

Röse Chloroform Absorption Method.[76] The method is based on the observation that



Courtesy of U. S. Dept. Agr., Bur. Chem. 1908, Bul. **107** *rev., p. 97*

FIG. 150. Bromwell Fusel Oil Tester.

chloroform, on shaking with a distilled liquor, increases in volume to an extent dependent on the content of fusel oil. The procedure has been modified by Herzfeld,[77] Windisch,[78] and particularly by the Swiss chemist Sell who has contributed calculation formulas and tables and has devised multiple apparatus.

The method, although not so accurate as the Allen-Marquardt method, serves as a rough test.

APPARATUS. The Herzfeld tester has been displaced in the United States by the more convenient form devised by Bromwell [79] with a stopcock at the lower end (Fig. 150).

PROCESS. *Purification.* Reflux the sample for 15 minutes to remove carbon dioxide, then distil 200 ml. with a little *potassium hydroxide* and dilute 160 ml. of the distillate to 200 ml. in a volumetric flask. Obtain the percentage of ethanol from the specific gravity in the usual manner.

Adjustment to 30% Alcohol Content. Dilute with water or fortify the distillate with pure absolute ethanol to 30% by volume (24.68% by weight), using the amounts calculated by Sell's formulas as follows:

$$10V - 300$$

and

$$X' = \frac{300 - 10V}{7}$$

in which X and X' are the milliliters of water or pure ethanol respectively required to secure an alcohol content of 30% by volume and V is the per cent by volume before adjustment.

Sell has published tables that give the volumes of water or ethanol to be added without calculation, which are here omitted, since the volumes are readily derived from the formulas if need arises.

After the dilution (or fortification), determine the specific gravity and adjust to exactly 0.96564 at 15°.

Agitation with Acid. Pour into the Röse-Herzfeld or Bromwell tester *chloroform* at 15° to the 20-ml. mark, add 100 ml. of the adjusted distillate and 1 ml. of *sulfuric acid*

(sp.gr. 1.2857), stopper, and cool to 15°. Sell uses a multiple mechanical shaker.

Reading. Remove from the bath, mix by slowly inverting, then shake vigorously but uniformily at least once a second for 2 minutes. Dislodge drops of chloroform from the sides of the tube, cool again to 15°, and read with a lens the height of the chloroform column to the lower meniscus.

Control. Carry along a control determination on pure absolute ethanol diluted to 30% by volume and deduct the reading (usually 1.64 ml.) from the reading of the unknown.

CALCULATION. Obtain the percentage of fusel oil from Sell's table herewith, which, although calculated for brandy, for practical purposes may be applied to other distilled liquors.

Calculate the results to the original liquor by the formula

$$X = \frac{F(100 + a)}{100}$$

in which X is the fusel oil content of the liquor in per cent by volume, F is the fusel oil from the tables, and a is the number of milliliters of water or ethanol added to 100 ml. to adjust to 30%.

FUSEL OIL FROM THE INCREASE IN VOLUME OF CHLOROFORM BY THE RÖSE METHOD (SELL)

Increase in Volume	Fusel Oil	Increase in Volume	Fusel Oil
0.02	0.0133	0.34	0.2255
.04	.0265	.36	.2387
.06	.0398	.38	.2520
.08	.0530	.40	.2652
.10	.0663	.42	.2785
.12	.0796	.44	.2918
.14	.0928	.46	.3050
.16	.1061	.48	.3183
.18	.1194	.50	.3316
.20	.1326	.52	.3448
.22	.1459	.54	.3581
.24	.1591	.56	.3713
.26	.1724	.58	.3846
.28	.1857	.60	.3979
.30	.1989	.62	.4111
.32	.2122	.64	.4244

Kilp and Lampe Modification for Liquors High in Fusel Oil.[80] PROCESS. Instead of diluting the distillate to 30% by volume, employ a measured aliquot of 10 ml. or more, according to the approximate amount of fusel oil believed to be present. Dilute the aliquot with 75 ml. of *fusel oil-free spirits,* then add *carbon dioxide-free water* until after thorough mixing the specific gravity is 0.965575 (24.68% by volume), taking care to avoid loss, since the final volume must be accurate to 0.5 ml. To compensate for the small loss sustained in spite of all precautions, add 1 ml. to the final volume. This correction was adopted by Kilp and Lampe after a large number of trials.

Employ 100 ml. of this liquid after the dilution to 30% in determining the increase in volume of the chloroform.

CALCULATION. Obtain the grams of fusel oil per 100 ml. of the sample (F) from the following equation:

$$\frac{Z \times 0.5428 \times V \times 100}{100 \times a}$$

$$Z \times 0.5428 \times V$$

in which Z is the increase in volume of the chloroform, V is the total volume of the solution, a is the aliquot in milliliters, and 0.5428 is the weight (in grams) of amyl alcohol (in 100 ml. of ethanol diluted to 30% by volume) corresponding to an increase in 1 ml. in volume of the chloroform as determined by Röse.

Allen-Marquardt-Schidrowitz Method.[81] The principle on which this method rests is the formation of valeric acid from amyl al-

cohol by oxidation. Fusel oil, however, contains in addition to amyl alcohol normal butyl, isobutyl, and propyl alcohol. Vasey states that amyl alcohol predominates in general in raw grain and potato spirits. In brandy half or two-thirds may be normal butyl alcohol. Straight whiskey may contain as high as 0.20% of fusel oil, whereas that made from neutral spirits, colored and flavored, may run as low as 0.02% or lower.

REAGENTS. *Purified Carbon Tetrachloride.* Shake well at intervals CCl_4 with one-tenth its volume of H_2SO_4 and allow to stand overnight. Wash with tap water until neutral, add NaOH solution in excess, and distil.

Oxidizing Mixture. Dissolve 100 g. of pulverized $K_2Cr_2O_7$ in 900 ml. of water and 100 ml. of H_2SO_4.

PROCESS. *Saponification and Distillation.* Saponify 100 ml. of the sample with 20 ml. of *0.5 N sodium hydroxide solution* by refluxing for 1 hour or allowing to stand overnight. Attach to a condenser and distil 90 ml., then add 25 ml. of water and distil further until 25 ml. additional of the distillate pass over.

(If aldehydes are present in excess of 15 parts per 100,000, add to the distillate 0.5 g. of *m-phenylenediamine hydrochloride,* boil under a reflux condenser for 1 hour, distil 100 ml., add 25 ml. of water, and continue the distillation until an additional 25 ml. is collected.)

Saturate approximately the distillate with *powdered sodium chloride* and adjust the specific gravity to 1.10 by adding a *saturated sodium chloride solution.*

Tetrachloride Extraction. Shake with successive portions of 40, 30, 20, and 10 ml. of *purified carbon tentrachloride* and wash the combined tetrachloride solution three times with 50-ml. portions of *saturated sodium chloride solution* and twice with 50-ml. portions of *saturated sodium sulfate solution.*

Oxidation. Transfer the tetrachloride solution to a flask containing 50 ml. of *oxidizing mixture* accurately measured and reflux for 8 hours to oxidize the fusel oil, using all-glass connections or corks covered with tinfoil for this step and the subsequent distillations.

Distillation. Dilute with 100 ml. of water, distil until only about 50 ml. remain, add 50 ml. of water, and again distil until only about 35 to 50 ml. of solution remain.

Titration. Combine the distillates and titrate with *standard 0.1 N sodium hydroxide solution,* using *phenolphthalein indicator.*

CALCULATION. Use the formula: 1 ml. of 0.1 *N* NaOH = 0.0088 g. of amyl alcohol.

Blank. Make a blank determination on 100 ml. of carbon tetrachloride beginning with the washing with sodium chloride and sodium sulfate.

NOTE. Opinions differ as to whether or not the acidity represented by the first titration, which some attribute to hydrochloric acid and others to fatty acids, should be included in the calculation of fusel oil. It is commonly included if less than 10% of the total acidity.

Tolman and Hillyer Modification.[82] After oxidation as carried out in the original method, but with the precaution of avoiding "spots of bichromate" on the flask by shaking and overheating by slow boiling over several thicknesses of asbestos board, proceed as follows.

Iodide-Thiosulfate Treatment. Draw off the oxidizing mixture from the tetrachloride in a separatory funnel and wash until all the bichromate is removed. Dilute the combined bichromate solutions up to 500 ml. in a volumetric flask and remove 200 ml. to a liter flask. Add 20 ml. of *hydrochloric acid,* 100 ml. of *1 + 1 potassium iodide,* and, from a buret, exactly 50 ml. of approximately *0.75 N sodium thiosulfate solution* (not standardized) or a smaller amount if the fusel oil is abnormally high.

Titration. Titrate the excess of bichromate with standard *0.1 N thiosulfate solution.*

Blank. Make a blank determination beginning with the washing of the tetrachloride with sodium chloride in the same manner as the actual analysis.

CALCULATION. Subtract from the number of milliliters of the 0.1 *N* thiosulfate solution used in the blank the number used in the actual analysis. The difference represents the amount of bichromate reduced by higher alcohols. Multiply this difference by 0.001773 to obtain the grams of higher alcohols.

Komarowsky-von Fellenberg Salicylic Anhydride Colorimetric Method;[83] **Budagjan and Iwanowa Modification.**[84] APPARATUS. *Colorimeter.*

REAGENTS. *Silver Nitrate Solution, N.* Dissolve 16.9 to 17.9 g. of $AgNO_3$ crystals in water and dilute to 100 ml.

Ethanol, 94 to 98%, free from aldehydes and fusel oil; also 50% prepared by dilution of each 100 ml. with water, in amount depending on the percentage of ethanol (Brix), as follows: 94%, 93.9 ml.; 95%, 95.9 ml.; 96%, 98.1 ml.; 97%, 100.4 ml.; and 98%, 102.7 ml.

Salicylic Anhydride Solution, 1%, in aldehyde- and fusel oil-free ethanol.

Standard Acetaldehyde Solutions, containing 0.0005 to 0.003 volume per cent in 50% purified ethanol.

Mohler Reagent. Dissolve 1 g. of fuchsin in 1 liter of water and mix 30 ml. of the solution with 20 ml. of $NaHSO_4$ solution (sp.gr. 1.308).

Standard Amyl Alcohol Solutions, containing 0.001 to 0.018 volume per cent in 50% purified ethanol.

Salicylaldehyde Solution, 1%. Dissolve 1 ml. of the aldehyde in 100 ml. of purified ethanol.

PROCESS. *Distillation.* Pipet 200 ml. of the sample into a flask, distil into a 200-ml. volumetric flask, make up to the mark, mix, determine the specific gravity at 15° and adjust to exactly 50%. If water is required, to obtain the number of milliliters, multiply the volume per cent of ethanol by 2, and subtract 100 from the product; if ethanol is required, subtract from 100 the volume per cent of ethanol multiplied by 2.

Silver Nitrate Treatment. To 100 ml. of the distillate, add 0.2 ml. of *62% sulfuric acid,* and neutralize to *phenolphthalein* with *30% potassium hydroxide solution,* then add 1 ml. additional and 2 ml. of *1.0 N silver nitrate solution.* Reflux for 30 minutes and distil until 100 ml. of distillate are obtained, then determine in this distillate aldehydes and fusel oil as follows.

A. Acetaldehyde. Color Formation. To one of 7 glass-stoppered 20-ml. colorimeter tubes, add 10 ml. of the distillate, to 4 others add 10 ml. of solutions containing respectively 0.0005, 0.0010, 0.0015, and 0.002% of *acetaldehyde,* then add 4 ml. of *Mohler reagent* to all the tubes, close with glass stoppers, and shake.

Color Comparison. After 20 minutes, match the color of the unknown with one of the knowns, interpolating if necessary.

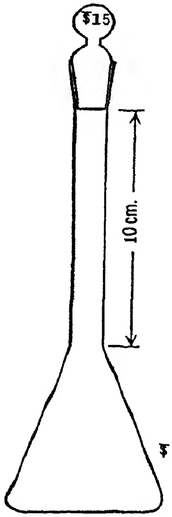
B. Fusel Oil. Color Formation. Into a 100-ml. volumetric flask pipet 10 ml. of the distillate; into 6 other 100-ml. volumetric flasks pipet 5 ml. of *standard acetaldehyde solution* of double the strength of the distillate, then 5 ml. of *standard amyl alcohol solution* in ascending series from 0.001 to 0.018 volume per cent, the amyl alcohol content of the mixture being of half concentration (0.0005 to 0.009%). Add to each of the 7 flasks 1 ml. of *ethanolic 1% salicylaldehyde solution* and, cautiously with the tube inclined, 25 ml. of *sulfuric acid.* After the reagents have been added to the entire series, mix immediately by rapid rotation.

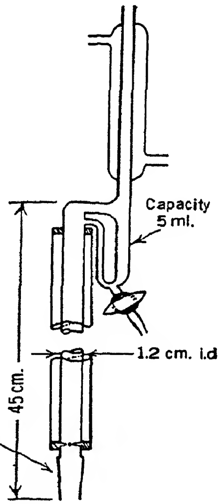
Color Reading. After 20 minutes, match the color of the unknown against the nearest member of the series, interpolating for greater accuracy.

Calculation. Reduce the reading to the basis of absolute ethanol by the formula

$$D \times V$$

in which X is the fusel oil content of the sample in per cent by volume calculated in terms of absolute ethanol, D is the fusel oil in the





Courtesy of J. Assoc. Official Agr. Chem. 1940, 23, 369

Fig 151. Schicktanz, Etienne, and Young Reaction Flask.

Fig. 152. Schicktanz, Etienne, and Young Dehydrating and Dealcoholizing Still.

50% distillate by comparison with the standards, V is the total volume of the distillate of the sample after adjustment to 50% by volume (volume of distillate + volume of ethanol required to obtain 50% by volume), and C is the original concentration of the distillate of the sample in per cent by volume (Brix).

Note. If the fusel oil exceeds 0.009% (this may be true of certain types of rum and cognac), dilute the distillate of the sample five-fold with 50% purified ethanol.

Schicktanz-Etienne Acetyl Chloride Volumetric Method; [85] **Schicktanz, Etienne, and Young Modification.**[86] The procedure was developed in the Alcohol Tax Unit Laboratory of the U. S. Treasury to give more consistent results in routine work. The percentage of fusel oil extracted with carbon tetrachloride depends on both the concentration and the volume of the saturated sodium chloride layer. It was thus found necessary to use a standard solution of the sample made up to a predetermined proof instead of an arbitrary 50 ml. independent of the proof. The volume taken is such that when made up to 50 ml. with water the proof will be about 90°; thus of a 100° sample only 45 ml. are used as calculated by the formula

$$\frac{4500}{\text{proof}} = x$$

in which x is the milliliters required to make 50 ml. of 90° liquid.

Apparatus. *Distillation Unit,* as used for the Allen-Marquardt method.

Reaction Flask (Fig. 151). A 125-ml. Erlenmeyer flask with elongated neck and standard-taper ground joint No. 15.

Dehydrating and Dealcoholizing Still (Fig. 152), packed with small glass helixes 0.64 cm. in diameter.

Reagent. *Acetyl Chloride,* Eastman's reagent grade. Used for preparing a 0.23 *M* solution in dry toluene.

Process. *Distillation.* Pipet the required quantity of the sample into the 500-ml. Erlenmeyer flask, add water to 50 ml., then 30 ml. *0.1 N alkali,* and a few *boiling stones.* Attach to the distillation unit and distil 50 ml. into a 125-ml. separatory funnel. Add 25 ml. of water to the Erlenmeyer flask and continue the distillation until the total volume of the distillate is 75 ml. The alcoholic concentrate and volume of distillate are in all instances the same.

Carbon Tetrachloride Extraction. To the

distillate in the separatory funnel, add 14 g. of *sodium chloride*, and shake for 1 minute. Extract with 40-, 30-, 20-, and 10-ml. portions of *carbon tetrachloride*, shaking for 1 minute after each addition. Collect the extract in the reaction flask containing a few bits of *carborundum* as anti-bump. Attach to the still and collect 50 ml. of distillate by allowing the still to reflux for 5 minutes before removing the first fraction, then removing nine more fractions at 5-minute intervals. Allow the reaction flask to cool 1 minute, then remove, loosely stopper, and cool 3 to 5 minutes in an ice bath.

Esterification. Add well down into the flask from a precision pipet 10 ml. of *pyridine solution* (approximately 0.5 *M* in toluene) and 20 ml. of *acetyl chloride solution* and tightly stopper, using a very small amount of lubricant. Shake and heat in a water bath [87] kept at 60° for 30 minutes, shaking every 5 minutes, then cool in an ice bath for 5 minutes and dilute with 25 ml. of water added so as to wash down the neck of the flask.

Titration. Transfer the liquid to a 500-ml. Erlenmeyer flask and add 100 ml. of *standard 0.1 N alkali* from a standard pipet. Shake well and then back-titrate with *standard 0.1 N sulfuric acid*, using *phenolphthalein indicator*.

Blank. Run a blank determination with each set.

CALCULATION. Obtain the difference between the number of milliliters of alkali required for the blank and the sample and calculate the grams of fusel oil per 100,000 in terms of amyl alcohol; 1 ml. of 0.1 *N* sodium hydroxide solution = 0.0001 mole (0.0088 g.) of fusel oil or 17.6 g. of fusel oil per 100,000.

EXAMPLES. Results by the Allen-Marquardt and the acetyl chloride methods respectively follow: whiskey 115 to 284 and 96 to 250, Scotch 37 to 134 and 19 to 102, and brandy 59 to 229 and 54 to 196.

ACIDS

A.O.A.C. Method. Titrate 100 ml. of the sample or, if colored, a smaller quantity, diluted to 100 ml., with *0.1 N sodium hydroxide solution*, using *phenolphthalein indicator*.

Calculate as grams of acetic acid per 100 ml., using the factor 0.006.

Schicktanz and Blaisdell Glass Electrode Potentiometric Method.[88] Measure into a beaker a sufficient quantity of liquor and submerge the glass or calomel electrodes, stir, preferably mechanically, and take the initial *p*H reading; then run in *0.05 N sodium hydroxide solution* slowly to a *p*H of 6 to 6.5. Note the volume of alkali and continue the titration by small increments (0.5 to 1.0 ml.), noting the *p*H after each. Continue until the *p*H reaches at least 11.5. Plot the values for *p*H against the volume of alkali between *p*H 6 and 11.5 for calculating the values for total, fixed, and volatile acids.

ESTERS

Tolman Saponification-Titration Method.[89] PROCESS. *Distillation.* Measure 200 ml. of the sample at 20° and dilute with 25 ml. of water. Distil slowly into a 200-ml. volumetric flask until nearly filled to the mark, using a mercury valve to prevent loss of ethanol. Adjust to 20°, add water to the mark, shake, and remove 50 ml. with a pipet, reserving the remainder for the determination of aldehydes and furfural.

Saponification. Neutralize accurately the 50-ml. aliquot with *standard 0.1 N sodium hydroxide solution*, using *phenolphthalein indicator*, then add 25 to 50 ml. additional, recording the volume added. Saponify by refluxing for 1 hour, or by allowing to stand overnight in a stoppered flask and heating the next morning for 30 minutes below boiling under a reflux condenser.

Titration. After cooling, titrate with *standard 0.1 N acid*, using *phenolphthalein indicator*, and deduct from the number of milli-

liters (25 or 50) of standard alkali added the number of milliliters of standard acid required for the titration.

CALCULATION. Use the formula: 1 ml. of 0.01 N alkali = 0.0088 g. of ethyl acetate.

ALDEHYDES

Vasey Sulfite-Fuchsin Colorimetric Method.[90] REAGENTS. *Aldehyde-Free Ethanol.* Digest 1 liter of ethanol, redistilled over NaOH, with 2 to 3 g. of *m*-phenylenediamine hydrochloride at room temperature for several days or reflux on a steam bath for several hours. Distil slowly, rejecting the first 100 ml. and last 200 ml.

Sulfite-Fuchsin Reagent. To a solution of 0.1 g. of pure fuchsin in 100 ml. of water, add a solution of 1 g. of SO_2 in water, dilute to 200 ml., and allow to stand until colorless. The solution keeps but a few days.

Rocques Standard Acetaldehyde Solution.[91] Grind aldehyde ammonia in a mortar with several portions of ether, decanting after each. Dry first in a current of air and then over H_2SO_4 *in vacuo*. Prepare a solution of 1.386 g. of the purified aldehyde ammonia in 50 ml. of ethanol, add 22.7 ml. of N ethanolic sulfuric acid, make up to 100 ml. with ethanol, and add 0.8 ml. additional to compensate for the volume of the $(NH_4)_2SO_4$ precipitate. Shake, allow to stand 24 hours, and filter. For use, dilute each day 2 ml. of the stock solution to 100 ml. with 50% by volume ethanol.

PROCESS. Keep all reagents and make all tests at 15°. Dilute 5 to 10 ml. of the solution prepared for the determination of esters (see above) to 50 ml. with *aldehyde-free 50% by volume ethanol*, add 25 ml. of *sulfite-fuchsin reagent*, and allow to stand 15 minutes at 15° to develop the color. Compare with solutions prepared by diluting measured amounts of the standard solutions to the same volume and treated with the same amounts of the reagents as the unknown.

CALCULATION. Use the formula: 1 ml. of standard aldehyde solution = 0.0002 g. of acetaldehyde.

FURFURAL

Girard and Cuniasse Acid-Aniline Method;[92] **Tolman Modification.**[93] REAGENT. *Standard Furfural Solution.* Prepare a stock solution by dissolving exactly 1 g. of redistilled furfural in water and diluting to 100 ml. in a volumetric flask with furfural-free ethanol. Each day as needed, dilute 1 ml. of the stock solution, measured in an accurate pipet, with 50% furfural-free ethanol, to 100 ml. in a volumetric flask; 1 ml. contains 0.1 mg. of furfural.

PROCESS. Measure with an accurate pipet 10 or 20 ml. of the solution prepared as described under Esters, into a 50-ml. volumetric flask and make up to the mark with *furfural-free 50% ethanol*. Add 2 ml. of *colorless aniline* and 0.5 ml. of *hydrochloric acid* (sp.gr. 1.125), mix and allow to stand in a water bath at 15° for 15 minutes together with a series of standards of the same volume treated in like manner. Match the color of the unknown against the standards.

CALCULATION. Use the formula: 1 ml. of standard furfural solution = 0.1 mg. of furfural.

TANNINS

Rosenblatt and Peluso Photocolorimetric Method.[94] APPARATUS. *Hilger Medium Quartz Spectrograph*, equipped with a Spekker null-type photometer having two rectifier-type photoelectric cells balanced by external resistance through a galvanometer with resistance dial read directly in units of per cent transmission.

Tungsten Filament Lamp, 100 watt.

Glass Filters. One each of a matched pair of Jena UG2 1 mm.

Bakelite Absorption Cell, 2.5 cm., with circular windows.

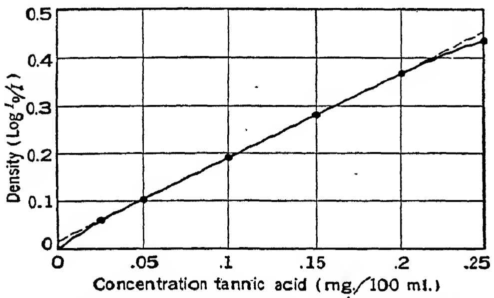
REAGENTS. *Folin and Denis Reagent.*

Reflux a solution of 100 g. of $Na_2WO_4 \cdot 2H_2O$, 20 g. of $20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$, and 50 ml. of 85% H_3PO_4 in 750 ml. of water for 2 hours, cool, to 25°, and dilute to 1 liter.

Sodium Carbonate Solution, saturated. Prepare from a supersaturated solution seeded at 25° several hours before use.

Tannic Acid Standard. Dissolve 0.100 g. of reagent grade tannic acid in water and dilute to 1 liter at 25°. Correct for moisture in the solid tannic acid.

PROCESS. *Calibration Curve.* Pipet 0.5, 1.0, 1.5, 2.0, and 2.5 ml. of *standard tannic acid solution* into each of five glass-stoppered 100-ml. volumetric flasks, each containing about 75 ml. of water, and 2 ml. of *Folin and Denis reagent*, then add 5 ml. of *saturated sodium carbonate solution*. Fill to the mark with water, shake well, and let stand for 1 hour at 25°.



Courtesy of J. Assoc. Official Agr. Chem. 1941, **24**, *177*

FIG. 153. Rosenblatt and Peluso Tannin Curve.

Prepare in like manner a blank containing all the reagents but no tannic acid. Use this blank to obtain the corrected zero value for the calibration curve when the photometer is adjusted to read 100% transmission with the blank in the cell. After an hour take the photometer readings, using red filter (Jena UG2 or KS64). Transform the transmission readings into density values (density = log $1/T$) and plot against concentration of tannic acid (Fig. 153).

Treatment of Sample. Pipet a volume of the sample, containing 0.05 to 0.25 ml. of tannins, into a 100-ml. glass-stoppered volumetric flask and proceed as with the standard, including a blank for the 100% transmission setting.

CALCULATION. Transform the transmission readings into density values and read the tannic acid content directly from the calibration graph.

EXAMPLES. Whiskey, 3 samples, 0.227 to 0.727 mg./ml.

COLORS

The coloring of whiskey made from neutral spirits is commonly caramel (burnt sugar). Prune juice primarily supplies flavor, but also contributes color. Flavescin and other coloring substances derived from oak wood of barrels are soluble in ether; caramel is insoluble.

Tentative A.O.A.C. Method for Water-Insoluble Color. Measure 50 ml. of the sample into a porcelain dish, evaporate to dryness but no longer, stir with 15 ml. of cold water, filter into a 50-ml. volumetric flask, and wash until the volume of the filtrate is nearly 25 ml. Add to the filtrate 25 ml. of *absolute ethanol* or 26.3 ml. of *ethanol*, make up to the mark with water, and mix.

Compare in a colorimeter with the original sample and calculate the percentage of color insoluble in water.

Marsh Amyl Alcohol Test. Bigelow [95] does not mention either amyl alcohol as a test for caramel or the Marsh test. Walker and Schreiber [96] merely state that C. T. N. Marsh observed that amyl alcohol removes the natural color of whiskey and not the caramel, from which it may be inferred that the original Marsh test is merely the amyl alcohol test.

I. Crampton and Tolman Modification.[97] The Tentative Marsh Test (under Artificial Colors) of the *A.O.A.C. Methods* is essentially this modification amplified by a test for coal-tar colors by shaking with an equal vol-

ume of fusel oil, amyl alcohol, or pentasol in which the coal-tar colors are insoluble.

REAGENT. *Marsh Reagent.* To 100 ml. of amyl alcohol, add 3 ml. of sirupy phosphoric acid and 3 ml. of water. Shake to form an emulsion before use.

PROCESS. To 5 ml. of the whiskey, add 10 ml. of the *reagent,* shake vigorously for a few minutes, and allow to separate. With pure whiskey, the lower layer will be perfectly colorless, but, if caramel, a coal-tar color, or an extract from uncharred oak chips is present, it will be colored and the relative amount of color in the two layers will furnish an indication of the amount of artificial color present.

II. Tolman and Hillyer Modification.[98] In the *Methods of the A.O.A.C.,* this test is described under the heading "Color Insoluble in Amyl Alcohol—Tentative."

REAGENT. *Marsh Reagent.* See method above.

PROCESS. Evaporate 50 ml. of the sample just to dryness, dissolve the residue in water, add 26.3 ml. of *ethanol,* and make up to 50 ml. with water. Shake 25 ml. of the mixture with 20 ml. of freshly shaken *Marsh reagent,* allow to separate, repeat twice, then draw off the lower layer completely into a 25-ml. cylinder, and dilute to 50 ml. with *50% by volume ethanol.*

Compare the color with 25 ml. of the untreated sample and calculate as percentage of color insoluble in amyl alcohol.

Mallory and Valaer Zinc Acetate Test for Caramel.[99] This is the Marsh Test (Tentative) of the A.O.A.C. Methods. In testing whiskey for artificial color, Mallory and Valaer suggest the following order: (1) Marsh test, (2) amyl alcohol test, and (3) zinc acetate test. The last named was developed to detect caramel in the presence of the reddish brown color extracted from uncharred oak chips present in some well-known brands.

PROCESS. Into a 150-ml. beaker with marks showing 13 and 25 ml., measure 25 ml. of the sample, add 0.5 ml. of *glacial acetic acid,* and mix, then add 0.75 g. of *zinc acetate crystals* and mix. Heat rapidly, with stirring, over a strong flame and boil vigorously with stirring until the volume is reduced to 13 ml., adjusting with water if necessary. Cool gradually, make up to 25 ml. with *ethanol,* mix, let stand several minutes, then mix again and filter through a 15-cm. pleated paper into a cylinder.

Treatment with Marsh Reagent. Mix the filtrate, pour 6 ml. into a 15-cm. test tube and add 12 ml. of *Marsh reagent.* Mix thoroughly, thus dissolving the heavy precipitate that first forms. Allow the layers to separate, pour off 4 ml. of the upper one, and replace by 4 ml. of *88% ethyl acetate.* Again mix thoroughly and allow the layers to separate.

The presence of color in the lower layer is indicative of caramel or a coal-tar dye. The latter is characterized by (1) a strong reddish shade, (2) a negative reaction with Marsh reagent, but a rather pinkish lower layer, and (3) a strong test with amyl alcohol. Add a few drops of *hydrochloric acid* to some of the zinc acetate filtrate. The pink color formed in the presence of certain coal-tar dyes is discharged by *stannous chloride solution.*

METHANOL

The presence of methanol (wood alcohol) in distilled liquors was not unusual during the era of prohibition when there was a strong incentive to rectify grain alcohol denatured with methanol. Since the distillation was carried on of necessity in hidden or remote plants by ignorant criminals without even the mechanical skill of safe-crackers, it is not remarkable that the product was a menace to life.

Riche and Hardy Methyl Aniline Violet Test.[100] Although somewhat lengthy, this qualitative test yields convincing results since it depends on the formation of methyl

aniline violet characterized by its intense color and affinity for woolen fibers.

Process. *Formation of Iodides.* Add 10 g. of the sample, previously rectified over potassium carbonate, if necessary—the significance of "necessary" is not clear—to 15 g. of *iodine* and 2 g. of *red phosphorus* contained in a small flask. Cool in ice water for 10 to 15 minutes or until action has ceased. Both ethanol and methanol are converted into iodides.

Aniline Treatment. Distil on a water bath, collecting the methyl and ethyl iodides in 30 ml. of water. Eliminate free iodine by washing with *dilute alkali* and draw off the oily liquor from the aqueous layer in a separatory funnel into a flask containing 5 ml. of *aniline*, cooling or warming according as the reaction is violent or sluggish.

Methyl Aniline Violet Formation. Let stand 1 hour, boil the product with water, and add 20 ml. of *15% sodium hydroxide solution.* As soon as the bases collect on the surface as an oily layer, fill the flask to the neck with water, pipet 1 ml. of the oily layer into a porcelain dish, and mix thoroughly with 10 g. of a mixture of 100 parts of *quartz*, 2 parts of *sodium chloride*, and 3 parts of *cupric nitrate.* Transfer to a large test tube, heat at 90° for 8 to 10 hours, exhaust with warm *ethanol*, filter, and dilute with *ethanol* to 100 ml.

Ethanol yields a liquid with a red tint, but in the presence of as little as 1% of methanol the liquid has a violet shade.

Dyeing Test. Dilute 5 ml. of the colored liquid to 100 ml. with water, then dilute 5 ml. of the diluted solution further to 400 ml. in a porcelain dish. Heat to boiling, add a piece of *white merino* (or nun's veiling or white woolen yarn) free from sulfur, and let stand for 30 minutes. If the liquor is pure, the woolen material will remain white but, if methanol is present, it will be dyed violet, varying in intensity according to the amount present.

Trillat Dichromate Dimethylaniline Test.[101] Process. *Oxidation.* Dilute 50 ml. of the sample with an equal volume of water, add 8 g. of *calcium oxide*, and distil fractionally through a Glinsky bulb tube. To the first 15 ml. of the distillate, diluted to 150 ml., add 15 g. of *potassium dichromate* and 70 ml. of *1 + 5 sulfuric acid.* Allow to stand for 1 hour with occasional shaking. Distil and reject the first 25 ml. which contains only acetaldehyde, then collect 100 ml.

Dimethylaniline Treatment. Transfer 50 ml. of the distillate to a flask of strong glass, add 1 ml. of *rectified dimethylaniline*, stopper tightly, and heat in a water bath at 70 to 80° for 3 hours with occasional shaking. Add *sodium hydroxide solution* to distinct alkalinity, distil until 25 ml. have passed over.

Color Reaction. To the residue in the flask, add *acetic acid* to acid reaction, mix 5 ml. with 5 drops of a suspension of 1 g. of *lead dioxide* in 100 ml. of water, and boil. If only grain alcohol is present in the sample, a blue color appears, changing immediately to yellow, then to green, and disappearing on boiling; if methanol is present, a blue color appears which increases in intensity on boiling.

Denigès Permanganate–Fuchsin Test. I. Kling and Lassieur Modification.[102] Reagent. *Schiff Reagent.* Add to 1 liter of a 0.1% solution of fuchsin 20 ml. of $NaHSO_3$ solution (sp.gr. 1.33). After 10 minutes, add 20 ml. of HCl and allow to stand until colorless.

Process. *Oxidation.* Dilute the sample to an alcoholic strength of about 1% and add to 2 ml. of the diluted solution 2 ml. of *2.5% potassium permanganate solution* and 0.4 ml. of *50% sulfuric acid.* After 3 minutes, destroy the excess of permanganate with *saturated oxalic acid solution.*

Color Formation. Add to the solution 1 ml. of *sulfuric acid* and 5 ml. of *Schiff reagent.* Owing to the action of formaldehyde on the decolorized fuchsin, in the presence of metha-

nol, a violet coloration is formed on standing.

One per cent of methanol produces a distinct and 3% a strong coloration. With higher concentrations the coloration is so intense as to make quantitative determinations impossible. An error due to the interfering color produced by ethanol is caused when a dilution greater than 1 : 100 is used.

II. Chapin Modification.[103] The technique of a qualitative procedure, devised in the U. S. Bureau of Animal Industry, is briefly as follows.

PROCESS. (1) Remove substances giving false reactions by distillation or in rare cases by special treatment; (2) add to 5 ml. of the distillate, diluted to a 5% by volume content of total alcohol, 0.3 ml. of *85% phosphoric acid* and mix; (3) add 2 ml. of *3% potassium permanganate solution*, mix, and let stand until the permanganate is entirely decomposed (10 minutes); (4) add 1 ml. of *10% oxalic acid*, mix, and let stand until the color becomes a clear brown (2 minutes); (5) add 1 ml. of *sulfuric acid* and mix; (6) add 5 ml. of *Schiff-Elvove reagent* (see modified Schiff reagent below), mix immediately, and let stand exactly 10 minutes. If methanol was absent or less than 0.2%, the color of the solution should be pale greenish without a distinct blue or violet tint.

QUANTITATIVE PROCEDURE. The details of the actual test for known and unknown involve the same amounts of the same reagents as for the qualitative method, but in the preparation of the solution the ethanol content is reduced by dilution to 1% of total alcohols, then twice diluted five-fold with 1% of pure ethanol.

III. Georgia and Morales Modification.[104] The Chapin modification is regarded by Georgia and Morales (Cornell University) as superior to previously described methods but dependable only as a negative test.

REAGENTS. *Potassium Permanganate Reagent.* Dissolve 3 g. of $KMnO_4$ in 15 ml. of 85% H_3PO_4 diluted with water, and dilute further to 100 ml.

Oxalic-Sulfuric Acid Mixture. Dissolve 5 g. of oxalic acid in 100 ml. of 1 + 1 H_2SO_4.

Modified Schiff Reagent. Dissolve 0.2 g. of rosaniline hydrochloride in 120 ml. of hot water, cool, add a solution of 2 g. of Na_2SO_3 in 20 ml. of water, and mix; then add 2 ml. of HCl, dilute to 200 ml., and store in well-filled glass-stoppered amber bottles. Elvolve uses 0.2 g. of fuchsin as the dye.

PROCESS. *Oxidation.* Dilute the distillate obtained in the determination of ethanol to an alcohol content of 5% by volume. Oxidize 5 ml. of the diluted liquid, contained in a test tube, for 10 minutes with 2 ml. of the *potassium permanganate reagent.* Destroy the excess of permanganate by the addition of 2 ml. of *oxalic-sulfuric acid mixture.*

Formaldehyde Test. When the solution becomes decolorized, add 5 ml. of *modified Schiff reagent*, mix, and note whether in 10 minutes the red-purple color characteristic of formaldehyde, formed from methanol in the process, appears.

Blanks. Run blanks on solutions of ethanol and methanol of the same dilution as that employed in the test.

IV. Beyer Modification.[105] As employed in the laboratory of the U. S. Internal Revenue Bureau, Washington.

APPARATUS. *Distillation Assembly* (Fig. 154).

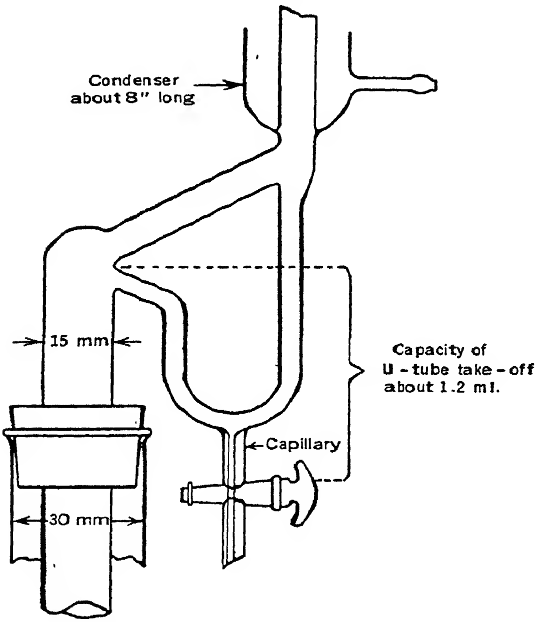
Nessler Tubes or *Photometer* with light filter 560 or 580 mμ.

REAGENTS. See foregoing modification.

PROCESS. *Distillation.* Place 25 ml. of the liquor, which may contain as little as 0.1% of methanol in 50% ethanol, in the 100 ml. round-bottom flask of the distillation assembly. Apply heat and when the distillation starts allow to remain under total reflux for about 30 minutes. Draw off through the capillary tube 1.2 ml. at 15-minute intervals until about 8.6 ml. are obtained. Transfer the combined portions to a 50-ml. cylinder or

volumetric flask and dilute to 22% total alcohol by volume, then further dilute with *22% ethanol* to 50 ml.

Oxidation. Place 4.75 ml. of water and 0.25 ml. of the diluted sample in a 15-mm. test tube (or 15-mm. Nessler tube if no photometer is available). Add 2 ml. of *phosphoric-permanganate reagent,* mix well with-



Courtesy of J. Assoc. Official Agr. Chem. 1939, **22,** *154*

FIG. 154. Beyer Methanol Distilling Assembly.

out inverting, and let stand 10 minutes with occasional shaking, then add 2 ml. of *oxalic-sulfuric acid reagent,* followed by 5 ml. of *modified Schiff reagent.* Mix well, inverting three times, stopper, and let stand about 1 hour if comparison is to be made in Nessler tubes or 2.25 hours if a photometer is used.

In the latter case, limit the number of simultaneous determinations to four, which, however, may be made every 20 minutes.

Reading. Compare the solution in the Nessler tubes with a series of standard solutions treated in like manner or make the reading in a photometer, using a 5-cm. cell and light filter 560 or 580 mμ and compare with a standard curve.

Filters 590 and 610 mμ give straight-line curves, but there is some difficulty in matching the two halves of the photometer.

Bono Dichromate Phenylhydrazine Test.[106] Although in its earlier stages the process is much the same as in the Trillat test, the later steps and the reagents for the final reaction are entirely different.

APPARATUS. *Distillation Train,* consisting of a distilling flask (*A*), a reaction flask (*B*), a vertical condenser (*C*), and a graduated receiver (*D*). *A* is closed by a double-bored stopper carrying a funnel-tube (*a*), with a stopcock, and a delivery tube passing to the bottom of *B*.

REAGENT. *Potassium Dichromate Reagent.* Saturate a solution containing 60 ml. of H_2SO_4 per liter with $K_2Cr_2O_7$ in the cold.

PROCESS. *Oxidation.* Place in flask *B* 50 ml. of *potassium dichromate reagent,* measure 25 ml. of the sample and 50 ml. of water into the 200-ml. flask *A* through the funnel tube *a,* boil, and collect the distillate in *D* containing the products formed in *B*. Reject the first 25 ml. containing only acetaldehyde, which is first formed by the oxidation of ethanol, then collect 25 ml. to be tested for formaldehyde formed by the oxidation of the methanol.

Formaldehyde Test. Mix 2 ml. of the second distillate with 10 drops of *0.5% phenylhydrazine hydrochloride solution,* 1 drop of *0.5% sodium nitroprusside solution,* and 10 drops of *10% sodium hydroxide solution.*

If methanol is present in the sample, the characteristic blue color appears, passing into green and then into yellow-red.

Leach and Lythgoe Immersion Refractometric Method.[107] In several respects the most satisfactory method for the detection [illegible]ntitative determination of methanol

in alcoholic liquors is by calculation from the Zeiss immersion refractometer reading in conjunction with the specific gravity or the percentage of alcohol derived therefrom. The original Leach and Lythgoe table gives the percentages of alcohol by weight and separate scale readings for methanol and ethanol.

A.O.A.C. Official Modification.[108] The revised table below gives specific gravity at 15.56°/15.56° instead of percentage of alcohol by weight and is in conformity with the latest Bureau of Standards tables for correlating specific gravity and refractive index with ethanol content.

PROCESS. Determine the ethanol by the distillation-density method and also take the refractometer reading at 17.5° of the distillate (see 1, Wine and Cider above). If, on consulting the tables the refractometer reading corresponds with practically the same percentage of ethanol as that from the specific gravity obtained in the ethanol determination, the absence of methanol is assumed; if they do not agree, find the scale readings for the two alcohols corresponding to the specific gravity obtained by interpolation in the table herewith.

CALCULATION. The calculation is best explained by an example: The distillate has a specific gravity at 15.56° of 0.9625 and a refractometer reading at 17.5° of 43.1. By interpolation in the table, the readings for ethanol and methanol corresponding to this gravity are 65.2 and 31.7 respectively and the difference is 33.5; $65.2 - 43.1 = 22.1$; $(22.1 \div 33.5) \times 100 = 66.0$, showing that 66.0% of the total alcohol present is methanol.

Sémichon and Flanzy Dichromate-Phenylhydrazine Colorimetric Method.[109] By first determining the oxidizability of the liquor, the yield of formaldehyde from methanol is increased from less than 10% of other authors to 32%. The procedure is made quantitative by subjecting a solution of known methanol content to the same treatment as the unknown and comparing, by the Bertrand method, the color of the two solutions with phenylhydrazine hydrochloride. The method was developed at the Enological Institute at Narbonne, France.

Either chromic trichloride or potassium dichromate (see Trillat Dichromate Dimethylaniline Test above) may be used for the oxidation as follows:

$$HCH_2OH + 2CrO_3 + 3H_2SO_4 \rightarrow Cr_2(SO_4)_3 + CO_2 + 5H_2O$$

$$HCH_2OH + K_2Cr_2O_7 + 4H_2SO_4 \rightarrow Cr_2(SO_4)_3 + K_2SO_4 + CO_2 + 6H_2O$$

APPARATUS. *Duboscq Colorimeter.*

REAGENT. *Acid Ferrous Ammonium Sulfate Solution.* In water dissolve 135.31 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, add 20 ml. of H_2SO_4, and dilute to 1 liter.

PROCESS. *Distillation.* Pipet 10 ml. of the liquor into a small distilling flask, add 50 ml. of water and a little *calcium carbonate,* then distil into a 50-ml. volumetric flask, make up to the mark, and mix. Designate the distillate as *A*.

1. QUALITATIVE TEST. *Preliminary Oxidation.* (*a*) *Distilled Liquors and Cordials.* Dilute 5 ml. of the distillate *A*, measured with a pipet, to 50 ml. in a second volumetric flask, pipet 5 ml. of the liquid thus diluted into a previously mixed and cooled mixture of 5 ml. each of *10% chromium trioxide* and *sulfuric acid,* and mix. After 15 minutes, titrate with *acid ferrous ammonium sulfate solution.*[110]

Calculate the total oxidizability (T) from the buret reading (t) by the formula

$$T = \frac{t}{3} \times \frac{50}{5} = \frac{10t}{3}$$

T is always less than 17 ml.

(*b*) *Wines and Malt Liquors.* Pipet 15 ml. of the distillate *A* without further dilution into a mixture of 20 ml. each of *4% potassium*

ETHANOL AND METHANOL IMMERSION REFRACTOMETER READINGS AT 17.5° C. FROM SPECIFIC GRAVITY (A.O.A.C.)

Sp.Gr. 15.56°/15.56°	Scale Reading 17.5°		Difference	Sp.Gr. 15.56°/15.56°	Scale Reading 17.5°		Difference
	Ethanol	Methanol			Ethanol	Methanol	
1.0000	15.0	15.0	0.0	0.9720	51.5	27.0	24.5
0.9990	15.8	15.3	0.5	.9710	53.0	27.5	25.5
.9980	16.6	15.6	1.0	.9700	54.6	28.1	26.5
.9970	17.5	15.9	1.6	.9690	56.1	28.7	27.4
.9960	18.5	16.2	2.3	.9680	57.6	29.2	28.4
.9950	19.4	16.5	2.9	.9670	59.1	29.6	29.5
.9940	20.4	16.9	3.5	.9660	60.6	30.1	30.5
.9930	21.4	17.2	4.2	.9650	62.0	30.6	31.4
.9920	22.5	17.5	5.0	.9640	63.3	31.0	32.3
.9910	23.6	17.9	5.7	.9630	64.6	31.5	33.1
.9900	24.7	18.2	6.5	.9620	65.8	31.9	33.9
.9890	25.9	18.6	7.3	.9610	67.0	32.4	34.6
.9880	27.1	19.0	8.1	.9600	68.1	32.8	35.3
.9870	28.4	19.5	8.9	.9590	69.2	33.3	35.9
.9860	29.6	19.9	9.7	.9580	70.2	33.7	36.5
.9850	31.0	20.4	10.6	.9570	71.2	34.1	37.1
.9840	32.4	20.8	11.6	.9560	72.1	34.5	37.6
.9830	33.8	21.3	12.5	.9550	73.0	34.9	38.1
.9820	35.2	21.8	13.4	.9540	73.8	35.3	38.5
.9810	36.7	22.3	14.4	.9530	74.6	35.6	39.0
.9800	38.3	22.8	15.5	.9520	75.4	35.9	39.5
.9790	39.9	23.4	16.5	.9510	76.2	36.2	40.0
.9780	41.5	24.0	17.5	.9500	76.9	36.5	40.4
.9770	43.1	24.5	18.6	.9490	77.6	36.8	40.8
.9760	44.8	25.0	19.8	.9480	78.3	37.0	41.3
.9750	46.5	25.5	21.0	.9470	79.0	37.3	41.7
.9740	48.2	26.0	22.2	.9460	79.7	37.6	42.1
.9730	49.8	26.5	23.3				

The scale readings are applicable only to instruments calibrated in the arbitrary scale units proposed by Pulfrich (*Z. angew. Chem.* 1899, p. 1168). According to this scale, 14.5 = 1.33300, 50.0 = 1.34650, and 100.0 = 1.36464. If the instrument used is calibrated in other arbitrary units, the refractive index corresponding to the observed reading can be converted into the equivalent Zeiss reading by referring to the St. John table.

dichromate and *sulfuric acid*, mix, and after 15 minutes titrate as above.

Calculate the total oxidizability (T) from the buret reading (t) by the formula

$$T = \frac{t}{3} \times \frac{15}{5} = t$$

T is always less than 20 ml.

Oxidation to Formaldehyde. Place in a 50-ml. flask $T/3$ ml. of the *chromium trioxide* or *potassium dichromate solution*, shake, then add 5 or 15 ml. respectively of solution A together with sufficient water to make a total volume of 35 ml. and 1 ml. of *sulfuric acid.* Shake vigorously and cool to 50° with constant agitation. After the liquid has changed successively to brown, then to green, and finally to blue, distil 5-, 10-, and 10-ml. fractions. The first fraction contains the unoxidized alcohol, the other two formaldehyde in quantities depending on, but not proportional to, the methanol content of the liquor.

Formaldehyde Test. Place in a test tube 5 ml. of the second fraction of the distillate, add 1 ml. of *freshly prepared 1% phenylhydrazine hydrochloride solution* and 5 ml. of *5% potassium ferricyanide solution,* and shake, then add 2.5 ml. of *hydrochloric acid.* The presence of formaldehyde is indicated by the formation of a red color due to fuchsin.

2. Quantitative Method. If the only alcohol present is methanol, employ for the determination of T the Duclaux method,[111] as above directed or the Zeisel method;[112] but if volatile substances other than methanol are present, employ only the Zeisel method.

If ethanol and methanol are present, proceed as follows.

After determining T, oxidize an accurately measured volume of the unknown (distillate A) and in the same manner an equal volume of accurately diluted *pure anhydrous methanol* corresponding approximately in methanol content to the unknown. Distil both unknown and known, collect three fractions of each, treat the second as directed above, and match the colors in a Duboscq colorimeter.

Calculation. Obtain the percentage of methanol (U) by the formula

in which K and U are respectively the percentages of methanol in the known and unknown and k and u are respectively the heights of the columns of the known and unknown.

Flanzy Iodization-Oxidation Method.[113] Flanzy not only considers that the formaldehyde method neither gives the full amount of methanol nor a definite fraction, but he also brands all existing methods as quantitatively unreliable. During the same year[114] he proposed a new method that is both tedious and complicated. The steps are (1) conversion of primary alcohols to iodides, (2) distillation of the alkyl halides, (3) determination of the iodization number by weighing the silver iodide formed with the distillate by silver acetate, and (4) determination of the oxidation number. After applying the method to numerous samples of fermented and distilled liquors, he reaches the surprising conclusion that methanol is a normal constituent of all natural alcoholic media.

Wilson Tetramethylammonium Iodide Gravimetric Method.[115] The apparatus is a modification of that employed by Zeisel and Fanto[116] for the determination of the methoxyl group, but iodine and red phosphorus are substituted for special constant-boiling hydriodic acid. Tetramethyl- and trimethylethyl-ammonium iodides, the former insoluble in absolute ethanol, the latter in 20 parts, are formed with trimethylamine in accordance with the following reactions:

$$(CH_3)_3N + CH_3I \longrightarrow (CH_3)_4NI$$

$$(CH_3)_3N +$$

Apparatus. *Distilling Assembly* (Fig. 155).

Reagents. *Trimethylamine Solution.* Cool with ice and salt, or in a refrigerator below 3°, 100 g. of anhydrous trimethylamine in a sealed container, 1 liter of absolute ethanol, and a 1-liter volumetric flask. Pour about 700 ml. of the absolute ethanol into the volumetric flask, followed by the trimethylamine, then rinse the container and make up to the mark with the cold absolute ethanol and mix. Gradually warm to room temperature and mix.

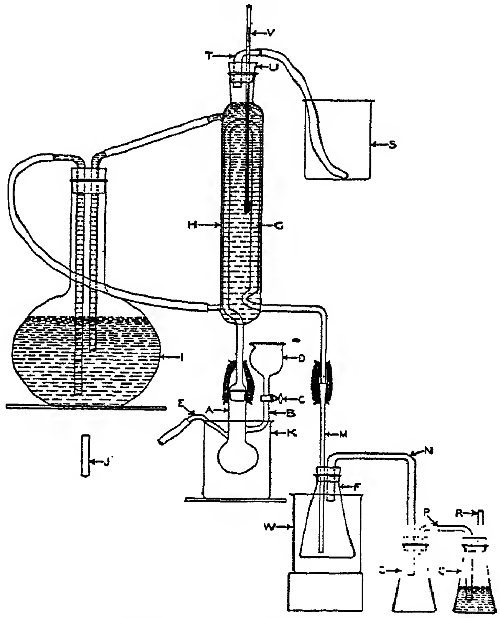
Wash Solution. Place in a 500-ml. flask about 0.25 g. of tetramethylammonium iodide from a previous determination, fill to a convenient height with absolute ethanol, stopper, and shake to saturate the liquid. Filter as needed through white ribbon filter paper.

Carbon Dioxide. Deliver from a tank fitted with a reducing valve and a rubber tube connected to the reaction flask (*A*).

Process. *Preliminary Distillation.* Distil slowly a portion of the sample, containing 20 to 25 ml. of absolute ethanol, and collect 50 ml. of distillate in a volumetric flask.

Distillation of Iodides. Raise the temperature of the water in *H* to 50 to 55° by a flame under *I*. Place 15 g. of *iodine* and 2 g. of *red phosphorus* in *A*, attach to *C*, and cool with ice and water in *K*. Introduce 2.5 ml. of *ethanol* into *A* through *D* and measure into *D* 10 to 20 ml. of the distillate of the sample containing not more than 0.160 g. of methanol nor 7 ml. of ethanol. Place 25 ml. of *wash solution* in *F*, connect with *G* by *M*, and cool in ice water in *W*. Attach the carbon dioxide tank to *E*. Stirring ice and water to keep as near 0° as possible, admit the distillate slowly through *C* so as to flow down the sides of *A* (3 to 5 minutes) and rinse the reservoir into *A* with 5 to 10 ml. of water in portions. Place water in *D* to prevent leakage and remove ice (but not water) from *K*. Allow the bottom dark red layer and the colorless layer to mingle gradually, adding if necessary ice to *K* and stirring to remove vapors of hydriodic acid that rise from the surface due to the violence of the reaction.

When the liquid becomes homogeneous, except for floating phosphorus, heat fairly rapidly to 75°, adding to *F* during the heating 10 ml. of *trimethylamine solution* and 25 ml. of *wash solution*, then attach flask *Q* containing



Courtesy of Methods of Analysis, A.O.A.C. 1935, p. 177

Fig. 155. Wilson Methanol Distilling Assembly.

dilute sulfuric acid to trap escaping trimethylamine. As soon as the contents of *A* begin to boil, deliver the *carbon dioxide* at about 50 bubbles per minute as counted in *W*. Continue the distillation 1.5 to 2 hours, maintaining the water in *K* at 75 to 80°, in *H* at 50 to 55°, and in *W* at or near 0°.

Titration. Disconnect *F*, rinse tube *M* with 10 to 15 ml. of *wash solution*, using a policeman if necessary, loosely stopper until the room temperature is reached, then close and let stand overnight. Filter on a tared sintered glass crucible (or Pregl tube for a

precipitate of less than 5 mg.), using 35 to 40 ml. of *wash solution* for the transfer. Ignore any clouds or crystals formed in the filtrate, since these are due to trimethylethylammonium iodide of which only 4 g. are soluble in 100 ml. of absolute ethanol. Wash off the outside of the crucible with *ethanol* to remove crystals of trimethylethylammonium iodide and suck dry. Turn off the suction, add 5 ml. of *wash solution* by pouring down the sides and mixing with the crystals, cover with a watch glass, let stand 2 or 3 minutes, and suck dry. After the third washing in this manner, remove the crucible from the holder, wash the outside carefully with *ethanol*, again suck dry, and dry at 100 to 110° for 1 hour. Cool in a desiccator and weigh.

CALCULATION. Use the formula: Weight of precipitate × 0.15933 = weight of methanol.

COPPER AND IRON

Callan and Henderson Carbamate Colorimetric Method. See Part I, C8*b*.

Gerber, Claassen, and Boruff Modification.[117] APPARATUS. *Spectrophotometer*, Coleman Universal.

REAGENTS. *Water*, redistilled in Pyrex

Bipyridine Reagent. Dissolve 0.2 g. of 2,2′-bipyridine in 100 ml. of 10% acetic acid. Ignore the pink color developing after a few hours.

Carbamate Solution, 1%. Dissolve 1 g. of sodium diethyldithiocarbamate in 100 ml. of water.

Hydroxyphenylglycine Reagent. Dissolve 0.1 g. of *p*-hydroxyphenylglycine in 100 ml. of 0.4 N H_2SO_4. Prepare daily.

Ammonium Acetate Solution, 1.0 N. Dissolve 77 g. of the salt in 1 liter of water and adjust to pH 6.

Standard Copper Solution. Dissolve 0.2 g. of pure copper wire in 15 ml. of 1 + 4 HNO_3 with warming. Boil to expel fumes, cool, and dilute to 200 ml. Prepare a ten-fold dilution. Just before use, dilute 10 ml. of the latter to 1 liter; 1 ml. = 1 γ.

Standard Iron Solution. Dissolve 0.2 g. of pure iron wire or electrolytic iron in about 10 ml. of 10% H_2SO_4, and dilute to 200 ml. Prepare two further dilutions as for copper.

Lead Nitrate Solution. Dissolve 1.6 g. of $Pb(NO_3)_2$ in water and dilute to 1 liter.

STANDARD CURVES. *Copper Standard.* Prepare copper standards containing 2 to 100 γ of copper in 20 ml. of solution, corresponding to 0.1 to 5 γ/g. Treat in the order given with 1 ml. of *hydroxyphenylglycine reagent*, 2 ml. of *bipyridine reagent*, 4 drops of *ammonium hydroxide*, 5 ml. of *1.0 N ammonium acetate solution*, and 1 ml. of *carbamate solution*. Extract each standard, in a separatory funnel with 15 ml. of *isoamyl alcohol*, this volume having been found sufficient to remove in one extraction all the carbamate complex formed by 100 γ of copper. Centrifuge at 2000 r.p.m. for a short time, separate the yellow alcohol layer and read the color value in the spectrophotometer against a blank solution incorporating all the reagents and steps except for the copper solution.

Iron Standard. Prepare also iron standards, running from 2 to 100 γ in 20 ml., and treat in the same manner as the copper standards. Make readings on the aqueous layer following a short centrifuging. When the concentration exceeds 60 γ in 20 ml. of bipyridine reagent (the bipyridine is the limiting factor), it is necessary to increase the strength of the color reagent to accommodate all the iron present.

Prepare copper carbamate and ferrous pyridine curves in a graph with 1 to 70 γ per 20 ml. as abscissas (*cl*), and 0 to 100 per cent of transmission (T), or optical density (D), that is $D_\lambda = \log 1/T$, as ordinates.

PROCESS. *Incineration.* Evaporate 100 ml. or more of the sample in a platinum dish and ignite in a muffle at dull redness for about 30 minutes. Take up the gray gossamer ash

in 1 ml. of *hydrochloric acid*, warm for a few minutes on the steam bath, and transfer with water to a 25-ml. volumetric flask, diluting to the mark.

Color Formation. Pipet 20 ml. to a separatory funnel and treat with reagents as described above for the standard curves.

Color Reading. Compare the yellow isoamyl alcohol solution and the pink aqueous solution against the corresponding layers of a blank.

CALCULATION. Obtain the concentration (K_λ) from the standard curve or from the formula

$$K_\lambda = \frac{\log 1/T}{cl} = \frac{D_\lambda}{cl}$$

in which T is the per cent of transmission, D_λ is the optical density (read directly from the Coleman spectrophotometer), c is the moles per liter, and l is the number of milliliters.

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G. DAIRY PRODUCTS

Nature and Importance of Dairy Science. The production and handling of milk, as well as the manufacture and standardization of milk products, which formerly were conducted in a haphazard manner, are now subject to chemical and bacteriological control. In placing the dairy industry on a scientific basis, the workers in experiment stations and factories have been active.

No other food industry demands such varied and exacting scientific work. The yearly milk and fat production of breeds and individual cows are determined, the milk of dairyman and vendor is inspected, and the great variety of manufactured milk products are subjected to continual scientific control. Still more exacting is the analysis of human milk and the constituents for the preparation of modified milk in conformity to physician's prescriptions. The content of the individual vitamins and enzymes as originally present or influenced by commercial practices, the kind and extent of metallic contamination due to conduits and containers, the wholesomeness of products as measured by bacterial count, and the presence of added water, preservatives, or colors or a deficiency in fat due to skimming are problems, varying from time to time in detail, that continually confront the scientific examiner.

1. MILK

The adult mammal, however different may be its natural preference, may exist on combinations of a great variety of foods, but the young of the species is more exacting, since it demands certain elusive growth factors as well as mere life factors. During the first year mother's milk is normally the one perfect food.

Milk of Different Animals. Although all young mammals demand essentially the same constituents in their milk, they thrive best on different proportions as indicated by the following general averages:

Composition of Milk of Different Animals

	Solids	Protein	Fat	Lactose	Ash	Samples
	%	%	%	%	%	
Woman *	11.7	1.19	3.11	7.18	0.21	67
Cow †	12.98	3.27	4.21	4.78	0.76	434 from various breeds
Goat ‡	13.12	3.76	4.07	4.44	0.85	67
Ewe §	19.30	6.09	7.43	4.81	0.97	From 8 ewes during 2 yrs.
Ass ‖	10.23	1.74	1.18	6.86	0.45	1
Mare ¶	9.87	1.65	0.94	6.98	0.30	—

* Elsdon: *Analyst* 1916, **41**, 74. † Lythgoe: *J. Ind. Eng. Chem.* 1914, **6**, 899. ‡ König: *Chemie Nahrungs- u. Genussmittel,* Berlin, 1903, **1**, 256. § *Golden and Puddy: J. Dairy Research* 1935, **6**, 307. ‖ Richmond: *Analyst* 1895, **21**, 88. ¶ Vieth: *Landw. Vers.-Sta.* 1885, **31**, 353.

As calculated by Bosworth and Van Slyke,[1] *cow's, goat's, and human milk* contains respectively: protein combined with calcium 3.200, 3.100, and 1.500, fat 3.900, 3.800, and 3.300, lactose 4.900, 4.500, and 6.500, dicalcium phosphate 0.175, 0.092, and 0.000, tricalcium phosphate 0.000, 0.062, and 0.000, calcium chloride 0.119, 0.115, and 0.059, monomagnesium phosphate 0.103, 0.000, and 0.027, dimagnesium phosphate 0.000, 0.068, and 0.000, trimagnesium phosphate 0.000, 0.024, and 0.000, sodium citrate 0.222, 0.000, and 0.055, sodium chloride 0.000, 0.095, and 0.000, potassium citrate 0.052, 0.250, and 0.103, monopotassium phosphate 0.000, 0.073, and 0.069, and dipotassium phosphate 0.230, 0.000, and 0.000%.

Colostrum. Cow's milk produced 2 or 3 days after calving, known as colostrum, differs from normal milk in that it contains about twice as much solids, owing chiefly to albumin (about 5%) and in lesser degree to ash (about twice normal), counterbalanced to some degree by a lower content of lactose (about half normal).

Milk of Different Breeds. Analytical data by Lythgoe [2] on the milk of 5 breeds appear in the following table.

COMPOSITION OF MILK OF DIFFERENT BREEDS OF COWS (LYTHGOE)

	Solids	Solids-Not-Fat	Protein	Fat	Lactose	Ash	Protein-Fat Ratio	Fat in Solids	Refraction at 20° C.		Ash of Sour Serum
									Copper serum	Sour serum	
	%	%	%	%	%	%		%			%
Jersey											
Min.	12.43	8.13	2.79	4.20	4.10	0.64	0.46	33.1	37.1	40.7	0.74
Max.	17.17	9.80	4.42	7.70	5.80	0.84	0.80	47.4	39.5	44.2	0.83
Av. (36)	**14.75**	**9.10**	**3.46**	**5.65**	**4.94**	**0.72**	**0.61**	**38.3**	**38.1**	**42.7**	**0.79**
Guernsey											
Min.	12.15	8.00	2.26	3.80	4.46	0.69	0.55	31.0	37.0	....	....
Max.	17.00	10.65	5.01	6.40	5.22	0.84	0.82	38.6	39.0	....	....
Av. (28)	**14.60**	**9.37**	**3.73**	**5.23**	**4.84**	**0.75**	**0.71**	**35.9**	**38.2**	....	....
Ayrshire											
Min.	11.44	7.89	2.22	3.30	4.05	0.58	0.59	27.2	36.0	38.7	0.78
Max.	14.68	9.46	3.91	5.40	5.30	0.87	0.92	36.3	38.8	42.7	0.92
Av. (27)	**12.64**	**8.63**	**2.99**	**4.01**	**4.88**	**0.76**	**0.75**	**31.8**	**37.7**	**40.5**	**0.86**
Dutch Belt											
Min.	10.93	7.63	2.34	3.00	4.20	0.63	0.71	26.3	37.3	....	....
Max.	14.09	9.43	3.61	4.75	5.35	0.77	0.97	31.8	39.0	....	....
Av. (41)	**12.15**	**8.59**	**2.96**	**3.56**	**4.93**	**0.70**	**0.83**	**30.9**	**38.3**	....	....
Holstein											
Min.	10.20	7.55	2.00	2.45	4.08	0.64	0.62	25.0	36.0	38.4	0.73
Max.	13.96	9.61	4.03	4.60	5.20	0.84	0.99	33.5	39.3	43.0	0.86
Av. (56)	**11.69**	**8.28**	**2.93**	**3.41**	**4.70**	**0.72**	**0.86**	**29.2**	**37.2**	**40.6**	**0.80**

Supplementing Lythgoe's figures are the results on fat and solids-not-fat respectively in the milk of single cows tested at the St. Louis Exposition in 1904, as given by Farrington:[3] Brown Swiss, 5 cows, 3.40 to 3.90, average **3.64** and 8.5 to 9.3, average **8.9**; Holstein-Friesian, 15 cows, 3.12 to 3.82, average **3.42** and 7.6 to 8.5, average **7.9**; Jersey, 25 cows, 3.85 to 5.08, average **4.67** and 8.42 to 9.01, average **8.75**; Shorthorn, 29 cows, 3.16 to 4.25, average **3.71** and 7.9 to 9.1, average **8.6**%.

Relative Value of Milk Constituents. It would be rash to emphasize the nutritional importance of any one constituent of milk, when by natural selection through the ages the proportion of each has reached a happy equilibrium. Commercially, however, the fat of milk is given the highest rating, whereas it is the protein of meat that takes precedence, fat meat being considered inferior. Milk fat, whether in cream or butter, stands first among animal fats, as does olive oil among vegetable oils. This is due largely to the agreeable flavor contributed by minor constituents of milk, augmented by diacetyl formed from acetyl methyl carbinol during the ripening of the cream. The carotene, forming the chief coloring constituent associated with the fat, gives butter fat special nutritional value, but this has no flavor itself, only an attractive color which serves as a lure.

Whatever the reason, milk is bought and sold according to its fat content, ignoring other nutritive constituents, but not of course ignoring constituents such as develop acidity or other undesirable flavoring constituents indicative of improper handling.

Of all the methods of analysis which follow, the determination of fat by the Babcock test is the most used and valued. Next in importance is the method of determining solids ("total solids") by evaporation and weighing the residue or calculation of the percentage from the fat and specific gravity. Protein, ash, and lactose are less often determined, their importance being in the order named. Lactose may be determined directly or by difference, subtracting the sum of the percentages of other constituents from 100.

Range in Composition. In addition to the species and breed, various factors influence the composition of milk. The fat generally increases as the period of lactation advances, and as the time between milkings decreases; also toward the end of each milking, following the same rule that causes fat to rise as cream in a container. Some authorities report a rise in fat content during the first few periods of lactation, others find the difference insignificant. Feeding, notwithstanding popular belief, may change the milk and fat yield, but not appreciably the fat content. So also gestation and oestrual period have little influence.

Standards. Because of the variation in the composition of milk due to natural causes, the fixing of municipal, state, and federal standards of composition has always been of questionable value in attaining the ends of justice; nevertheless it has persisted at the risk of prosecuting the innocent. The present U. S. Standards wisely do not give legal limits of composition. This does not mean that the guilty go free, but rather that verdicts are rendered in accordance with the usual rules of evidence, taking into account all the circumstances attending each case and not dogmatically comparing the analyst's results with the figures of a regulation.

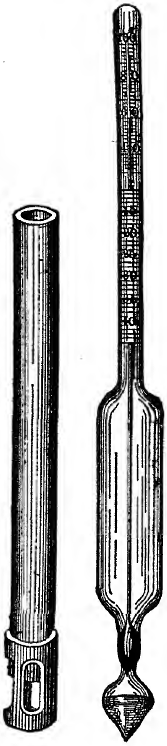
Sample

An analysis made on an improperly drawn sample of milk is particularly useless because of the rapid separation of the cream layer, which in a few hours reaches a fat content of 25% or higher, whereas the skim milk contains less than 1%. Thorough mixing of milk is, however, more readily carried out than that of powders or granular products containing ingredients of different specific gravity, such as bran and powdered endosperm. Pouring twice from one pail to another or stirring with a dipper is sufficient for the purpose. If in a bottle with a small air space, turning from end to end with shaking attains the same end.

Preservation of the Sample. Samples not tested within a few hours should be kept in a refrigerator or should be preserved with one of several suitable antiseptics.

Potassium bichromate (1 g. per quart) is

commonly used.[4] Winton and Ogden used for half-pint samples the contents of a 0.32-inch caliber pistol cartridge. Samples thus preserved, kept at room temperature for 2 give satisfactory results on fat and



A B

FIG. 156. *A*, Scovell Milk-Sampling Tube; *B*, Quevenne Lactometer.

solids, but not on acidity, physical constants, enzyme reactions, etc. Grelot [5] found that bichromate causes the formation of substances reacting like formaldehyde.

Mercuric chloride is a better preservative, but owing to its poisonous nature should not be used outside the laboratory and then only in conjunction with a coal-tar color. Grelot [6] recommends for half-pint samples tablets containing 0.05 g. of mercuric chloride and 0.0125 g. of ammonium chloride.

Formaldehyde (1 ml. of 40% solution per quart) is a good preservative, but it hardens the casein and interferes somewhat with fat determination by the Babcock test.

Caution! No preservative should be added unless previous experiment has shown that it not only keeps the sample, but also does not itself interfere with the analytical processes. In the determination of total solids and ash, a correction should be made for a non-volatile preservative.

Composite Samples. To avoid daily tests, whether in the commercial valuation or the official inspection of milk, samples collected from day to day are placed in a jar with a preservative and the composite sample is tested at the end of a week, 2 weeks, or even a month. In order that such a sample be fairly representative, the portion taken each day should bear the same volume ratio to the whole. This is automatically accomplished by using a Scovell sampling tube (Fig. 156*A*) which is inserted in the can of milk with the end open as in the cut. After being filled by lowering to the bottom of the can, the tube is closed by pressing down and the contents transferred to a fruit jar.

ADDED WATER

The purpose of certain milk tests and analytical methods is the detection of water fraudulently added. Given the same fat content, watered milk is a more serious menace to proper nutrition than skim milk, since it reduces non-fatty constituents by dilution. The double fraud of skimming and watering, skillfully proportioned, may result in a product with normal specific gravity, hence the need of additional physical tests and chemical methods interpreted in the light of accumulated data on milk of known purity.

Lactometer and creaming tests, supplemented by examination in rough-and-ready

apparatus such as the lactoscope and the pioscope, are merely preliminary and are not sufficient for court evidence. Of special value are the refraction and specific gravity of the milk serum and the still more convincing results on solids, fat, and (calculated) solids-not-fat.

Each inspection laboratory, whether official or commercial, follows methods approved by national organizations and carried out by trained chemists qualified by experience and knowledge of the literature.

Specific Gravity

The determination of specific gravity is not only the most valuable of all preliminary tests, but, coupled with the determination of fat by the Babcock method, it permits the calculation of the total solids or solids-not-fat with a degree of accuracy sufficient for most purposes.

The Westphal balance or any of the several forms of pycnometer gives accurate results, but for inspection purposes an accurately calibrated Quevenne lactometer (Fig. 156*B*) is satisfactory. It consists of a hydrometer with a special calibration that omits 1.0 from the specific gravity and gives the remaining numerals without a decimal point; thus, a specific gravity of 1.031 is equivalent to a Quevenne lactometer reading of 31.

Opacity

It is well known in the dairy industry that skimming increases and watering diminishes the specific gravity and that a skillful combination of the two cannot be detected by the lactometer. In order to combat the double fraud, two opacity tests, employing special apparatus, have come into use in Europe, although they are little known in the United States.

Feser Lactoscope Method. The simple principle utilized in this test is that dilution diminishes the opacity. The instrument consists of an outer glass cylinder graduated arbitrarily in percentage of fat, within which, centrally located at the bottom, is a small cylinder with black lines. By means of a pipet, 4 ml. of milk are measured into the outer cylinder, then water is added in portions with shaking until the opacity is sufficiently reduced to make the lines on the inner cylinder visible. The reading is taken at the surface of the diluted milk.

Heeren Pioscope Method. Instead of dilution of the sample, in this test a thin film is formed in a central chamber of a hard rubber disk beneath a cover-glass and the resultant tint of the milk film and black background is matched with a series of standard tints, radially arranged in the apparatus for ready comparison.

Freezing Point

The determination of freezing point, using the Beckmann apparatus [7] or the Hortvet cryoscope, although capable of wide application, is of particular value in detecting added water in milk. According to Wiss [8] A. Schmidt was the first to employ the freezing point for this purpose. The range for mixed herd's milk is usually well within the limits -0.57 and -0.53. It is a moot question whether the freezing point has any advantage over the refraction of the serum as a check on the fat and solids-not-fat in detecting watering. The apparatus costs less than the refractometer, but this is offset by the cost of the ether and the greater skill required in manipulation.

Hortvet Cryoscopic Method.[9] Apparatus. The *Cryoscope* shown in Fig. 157 is supplied by Eimer and Amend completely assembled together with instructions. *D* is a 1-liter Dewar flask 28 cm. high, enclosed in a metal casing, and having a four-hole cork through which pass (1) a central glass or metal tube 250 mm. long and 33 mm. outside diameter,

(2) a metal air inlet pipe with a perforated loop at the bottom connected with B, a Folin drying tube containing 10 to 15 ml. of sulfuric acid, (3) T a metal pipet with side tubulature through which ether vapor passes

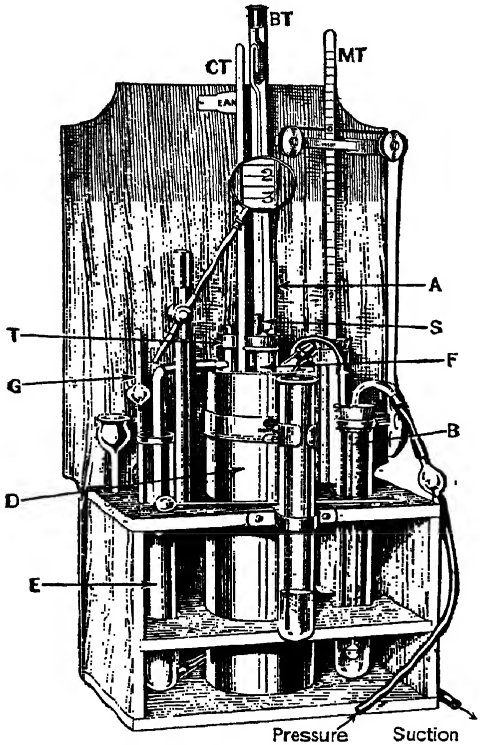


FIG. 157. Hortvet Cryoscope.

into E, a drain tube, and (4) CT a control thermometer.

Fitted closely into the 33-mm. tube is the freezing test tube into which through a triple-bored rubber stopper are fitted (1) BT a Beckmann thermometer for general use or a standard milk thermometer, (2) A a stirring device, and (3) S a tube for inserting a metal rod with a carrier for a small piece of ice to start freezing.

Other parts shown are G the ether gauge, MT the standard milk thermometer, with a range from $+1°$ to $-2°$ with tenths and hundredths divisions, fitted to an ether freezing point tube, and a Muencke pump for air pressure and suction.

PROCESS. *Procedure with Water.* Introduce into the Dewar flask (D), through a funnel tube inserted in T, 400 ml. of *ether* cooled to 10°, cork, connect the inlet tube with Folin drying tube (B), and run air through the apparatus 5 to 10 minutes until the control thermometer registers $-3°$, replacing the ether evaporated (10 to 15 ml.) as gauged by a glass tube lowered into the ether bath and closed with the finger tip.

Next add sufficient boiled water, cooled to 10° or lower, to the freezing tube to cover the thermometer bulb (30 to 35 ml.) which is then inserted together with the stirrer. Lower the freezing tube into the 33-mm. tube containing sufficient *ethanol* to fill the space between the tubes, using a section of thin-walled rubber tubing as a connecter. Keep the stirrer moving a stroke per 2 to 3 seconds, or more slowly while running the air current, until the temperature of the cooling bath reaches about $-2.5°$ and the standard thermometer shows a reading of about $-2.5°$ and a recession to about 0. Continue the cooling with stirring until the water is supercooled to 1.0 to 1.2°, when freezing accompanied by a rise in the mercury column begins. At this stage, when the mercury column approaches the highest point, stir slowly and tap the thermometer cautiously with a small cork mallet until the mercury remains constant about 2 minutes. Take the reading estimated in the third place of decimals, avoiding parallax. Duplicate the determination, then remove the thermometer and stirrer and pour the water out of the freezing tube.

Procedure with Sample. Rinse the tube with about 25 ml. of the sample cooled to 10°

or lower, measure into it 35 ml. of the sample or sufficient to submerge the thermometer bulb (*MT*), and insert the test tube in the 33-mm. tube. Keep the cooling bath at 2.5° below the probable freezing point of the sample and proceed with the determination as directed for water, with the difference that usually it is necessary to introduce a fragment of ice for the starter, when the mercury column has reached 1.2° below the probable freezing point. When, after a rapid rise in temperature, the mercury approaches its highest point, stir slowly and cautiously 2 or 3 times and take the reading.

Calculation. To obtain the freezing point depression of the milk, calculate the algebraic difference between the readings on the water and on the sample. Introduce a correction obtained in standardizing the thermometer as described below.

Examples. Although a standard milk thermometer of highest accuracy accompanies the apparatus, a correction should be applied from data secured by the analyst in observing the freezing point of boiled water and solutions of pure sucrose, obtainable from the Bureau of Standards, Washington, D. C., containing 7 and 10 g. per 100 ml., as illustrated in the following example, the details of which, together with certain foregoing data, were kindly furnished by the originator of the apparatus shortly before his death.

Observed Freezing Point: Water + 0.056, sucrose 7 g. −0.369, sucrose 10 g. −0.565, milk sample −0.471.

Freezing Point Depression: Sucrose 7 g., theory 0.422, found 0.425; sucrose 10 g., theory 0.621, found 0.621; milk sample 0.547.

Intervals: Sucrose 7 to 10 g., theory 0.199, found 1.96; sucrose 7 g. to milk sample, found 0.122, corrected $(0.199 \times 0.122) \div 0.196 = 0.124$.

Corrected Freezing Point Depression: Milk sample $0.422 + 0.124 = 0.546$.

Refraction of the Serum

Of the numerous methods of detecting added water in milk not requiring a chemical balance, yet based on scientific knowledge of the proportion of the milk constituents, the methods involving curdling and determination of the refraction of the serum by the immersion refractometer combine simplicity with accuracy. The apparatus is more expensive than that required for freezing point determination, but it is more easily manipulated and the cost for reagents is much less.

The leading refractometric methods which have been devised differ only in the preliminary separation of the serum which may be by natural souring or by adding reagents, such as copper sulfate. Woodman [10] uses 2 ml. of *25% acetic acid* to 100 ml. of milk, heating at 70° for 20 minutes, and Ackermann [11] 0.75 ml. of *calcium chloride solution* (sp.gr. 1.035) to 90 ml. of milk, heating on a boiling water bath for 15 minutes. Baier and Neumann [12] use equal volumes of milk and a solution of 30 g. of *asaprol* and 55.89 g. of *crystalline citric acid* in 1 liter of water without heating. Lythgoe adds a solution of copper sulfate as follows.

Lythgoe Copper Sulfate Method.[13] The advantages of this method are its simplicity and the narrow range of the results on pure milk, the latter feature contributing to its dependability as a forensic method. Specific gravity may also be determined on the filtered serum.

Apparatus. *Immersion Refractometer.*

Reagent. *Copper Sulfate Reagent.* Dissolve 72.5 g. of $CuSO_4 \cdot 5H_2O$ in water and dilute to 1 liter. Adjust to a refraction of 36° at 20°.

Process. To 4 volumes of milk, add 1 volume of *copper sulfate reagent*, shake well, and filter. Examine with the immersion refractometer the filtered serum, which should be at 20°, contained in one of the small beakers supplied with the apparatus. Fur-

ther details are given by the manufacturers of the refractometer.

EXAMPLES. Milk of individual thoroughbred Holsteins, Durhams, and Ayrshires showed refraction at 20° ranging from 36.3 to 39.7 and specific gravity at 20°/4° from 25.0 to 28.2. The corresponding figures on the natural sour serum were 38.6 to 44.4 and 23.4 to 29.2, but it should be remembered in making comparisons that in normal souring there is no dilution, whereas the addition of the copper sulfate reagent causes a dilution of 20%. Other results are given in the table at the beginning of the chapter.

SEDIMENT

Gerber Test.[14] The apparatus consists of a cylinder with a funnel-shaped bottom to which is attached by a rubber connection a narrow graduated tube for collecting the sediment.

Lobeck Test.[14] Instead of a graduated tube, a cotton wool filter held in place by a wire gauze disk, in a mounting similar to that of a rubber-stoppered beer bottle, serves to collect the sediment. The milk is heated to 30 to 35°.

Lorenz Test.[15] The apparatus consists of a cylinder with a funnel-shaped bottom, over which fits a brass cap, containing a wire gauze circle and cotton disk, held in place by a wire clamp. The cylinder is provided with a jacket so that hot water may be used to accelerate the filtering. This apparatus, set up at the intake of a distributing center, creamery, or cheese factory, furnishes ocular evidence to each patron of the cleanliness of his milk.

Centrifugal Test. A centrifuge with carriers for graduated sediment tubes of the type designed for blood, urine, and sputum tests may be used for separating dirt. In making comparisons, the machine should always be run at the same speed. The deposit may be measured in the graduated tube or, after washing and drying, weighed on a Gooch crucible.

LEUCOCYTE COUNT

Doane and Buckley Method.[16] Centrifuge 10 ml. of the milk for 5 minutes in a graduated sedimentation tube at a speed of approximately 2000 r.p.m. Remove the fat by means of absorbent cotton and siphon the milk down to 0.5 ml. Add 2 drops of an *ethanolic methylene blue solution* to the sediment and thoroughly mix by shaking, then place the tube in boiling water for 2 minutes. Make up the volume to 1 ml. with water and count the actual number of leucocyte cells in a Thoma-Zeiss counting chamber, using a 1/6 objective. Calculate the number of leucocyte cells per milliliter.

SOLIDS

The term total solids, so often used, suggests a summation of individually determined constituents, but in fact it is applied to the result of a single determination. The authors who insist on its use inconsistently omit it from water, fat, and ash. The total seems as superfluous as the crude of crude fiber, referred to elsewhere, when water, protein, fat, and ash are in the same sense crude.

Provided the dried residue is not used subsequently for the fat determination, the most satisfactory method for the determination of solids is the dish method in which the charge is evaporated and dried in an open dish at the temperature of boiling water. Other methods designed for the subsequent determination of fat employ asbestos or sand for the purpose of distributing the solids, thus permitting ready access of the ether to the fat.

Dish Gravimetric Method. It was long thought that water could not be thoroughly removed by direct drying of milk without

addition of an absorbent such as sand, asbestos, or filter paper, as described below. Later direct evaporation was deemed sufficient and in the laboratory of the Massachusetts Board of Health even the drying in a boiling water oven after evaporation on the steam bath is omitted.

PROCESS. Pipet into a tared flat-bottom dish, preferably of metal, 5 cm. in diameter, 5 ml. of the sample, or a volume representing 5 g. of average composition, and weigh quickly. Evaporate to dryness on a water bath and dry to constant weight in a boiling water oven.

A platinum dish permits determination of ash in the same portion. Tinned lead caps for wide-mouth bottles 2½ inches (6.3 cm.) in diameter and about 1 inch (2.6 cm.) high are so inexpensive that they may be thrown away after using.

Asbestos Gravimetric Method. This, like the sand method, has the advantage that fat may be determined in the same portion by ether extraction. Ignite for 1 to 2 minutes 5 g. of woolly *asbestos*, free from fine or brittle material, and introduce while hot into a dish such as is suitable for the foregoing method or into a Babcock perforated metal cylinder (Fig. 158) 6 cm. high and 2 cm. in diameter, closed near the bottom with a disk of the same material. Cool in a desiccator, weigh, add 5 ml., or a volume equivalent to 5 g., of the sample, as described above, and weigh without delay. Evaporate the water on a boiling water bath or in a water oven with the door open, completing the drying to constant weight in the oven with the door closed.

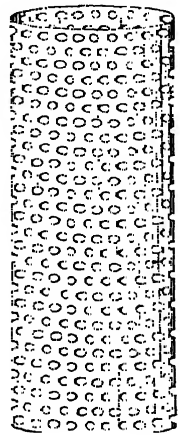
Calculate the percentage of solids and reserve the dish or cylinder with solids for fat determination.

Sand Gravimetric Method. Ignite 15 to 20 g. of *sand*, introduce into a dish, such as is used for the dish method, together with a short stirring rod, cool in a desiccator, and weigh. Add 5 ml. (or a volume approximately equivalent to 5 g.) of the sample and weigh. Evaporate with stirring and dry to constant weight.

Calculate the percentage of solids and reserve the dried residue for fat determination.

In this, as well as in the asbestos method, the tinned lead dish has the advantage that it may be crumpled up and introduced into the extractor for subsequent fat determination.

Adams Paper Coil Gravimetric Method. In this English method for the determination of solids and fat in one weighed portion, a strip, 6 cm. wide and about 60 cm. long, of fat-free porous paper is rolled into a coil and fastened with fine wire or cotton thread. The paper for the method is not always fat-free and must be carefully extracted by the analyst. Richmond[17] points out certain other defects of the method. In the United States it has not proved equal to the preceding methods and now is little used.



158. Babcock Perforated Milk Absorption Cylinder.

PROCESS. Place the *paper coil* in a glass-stoppered weighing bottle and dry to constant weight in a boiling water oven. Add to the coil from a pipet 5 ml. (or a volume approximately equivalent to 5 g.) of milk and weigh again. Suspend the coil in a warm

place until the bulk of the moisture has evaporated, then return it to the bottle and dry as before to constant weight.

Babcock Formula Method. Up to the time when the Babcock centrifugal method was introduced and only gravimetric methods for the determination of fat were dependable, it was customary to determine the percentage of solids, by one of the methods above described, also the specific gravity, then to calculate the percentage of fat from these data. The reverse procedure of determining the fat by the Babcock method and specific gravity, then obtaining the solids by one of several formulas or reference to a table now is practicable and has the advantage of dispensing with the chemical balance.

Determine the fat by the Babcock centrifugal method and the lactometer reading at

TEMPERATURE CORRECTION TABLE FOR LACTOMETER READING (VIETH)

Lactometer Reading	Degrees Fahrenheit														
	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
20	19.0	19.0	19.1	19.1	19.2	19.2	19.3	19.4	19.4	19.5	19.6	19.7	19.8	19.9	19.9
21	19.9	20.0	20.0	20.1	20.2	20.2	20.3	20.3	20.4	20.5	20.6	20.7	20.8	20.9	20.9
22	20.9	21.0	21.0	21.1	21.2	21.2	21.3	21.3	21.4	21.5	21.6	21.7	21.8	21.9	21.9
23	21.9	22.0	22.0	22.1	22.2	22.2	22.3	22.3	22.4	22.5	22.6	22.7	22.8	22.8	22.9
24	22.9	22.9	23.0	23.1	23.2	23.2	23.3	23.3	23.4	23.5	23.6	23.6	23.7	23.8	23.9
25	23.8	23.9	24.0	24.0	24.1	24.1	24.2	24.3	24.4	24.5	24.6	24.6	24.7	24.8	24.9
26	24.8	24.9	24.9	25.0	25.1	25.1	25.2	25.2	25.3	25.4	25.5	25.6	25.7	25.8	25.9
27	25.8	25.9	25.9	26.0	26.1	26.1	26.2	26.2	26.3	26.4	26.5	26.6	26.7	26.8	26.9
28	26.7	26.8	26.8	26.9	27.0	27.0	27.1	27.2	27.3	27.4	27.5	27.6	27.7	27.8	27.9
29	27.7	27.8	27.8	27.9	28.0	28.0	28.1	28.2	28.3	28.4	28.5	28.6	28.7	28.8	28.9
30	28.6	28.7	28.7	28.8	28.9	29.0	29.1	29.1	29.2	29.3	29.4	29.6	29.7	29.8	29.9
31	29.5	29.6	29.6	29.7	29.8	29.9	30.0	30.1	30.2	30.3	30.4	30.5	30.6	30.8	30.9
32	30.4	30.5	30.5	30.6	30.7	30.9	31.0	31.1	31.2	31.3	31.4	31.5	31.6	31.7	31.9
33	31.3	31.4	31.4	31.5	31.6	31.8	31.9	32.0	32.1	32.3	32.4	32.5	32.6	32.7	32.9
34	32.2	32.3	32.3	34.4	32.5	32.7	32.9	33.0	33.1	33.2	33.3	33.5	33.6	33.7	33.9
35	33.0	33.1	33.2	33.4	33.5	33.6	33.8	33.9	34.0	34.2	34.3	34.5	34.6	34.7	34.9

Lactometer Reading	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
20	20.1	20.2	20.2	20.3	20.4	20.5	20.6	20.7	20.9	21.0	21.1	21.2	21.3	21.5	21.6
21	21.1	21.2	21.3	21.4	21.5	21.6	21.7	21.8	22.0	22.1	22.2	22.3	22.4	22.5	22.6
22	22.1	22.2	22.3	22.4	22.5	22.6	22.7	22.8	23.0	23.1	23.2	23.3	23.4	23.5	23.7
23	23.1	23.2	23.3	23.4	23.5	23.6	23.7	23.8	24.0	24.1	24.2	24.3	24.4	24.6	24.7
24	24.1	24.2	24.3	24.4	24.5	24.6	24.7	24.9	25.0	25.1	25.2	25.3	25.5	25.6	25.7
25	25.1	25.2	25.3	25.4	25.5	25.6	25.7	25.9	26.0	26.1	26.2	26.4	26.5	26.6	26.8
26	26.1	26.2	26.3	26.5	26.6	26.7	26.8	27.0	27.1	27.2	27.3	27.4	27.5	27.7	27.8
27	27.1	27.3	27.4	27.5	27.6	27.7	27.8	28.0	28.1	28.2	28.3	28.4	28.6	28.7	28.9
28	28.1	28.3	28.4	28.5	28.6	28.7	28.8	29.0	29.1	29.2	29.4	29.5	29.7	29.8	29.9
29	29.1	29.3	29.4	29.5	29.6	29.8	29.9	30.1	30.2	30.3	30.4	30.5	30.7	30.9	31.0
30	30.1	30.3	30.4	30.5	30.7	30.8	30.9	31.1	31.2	31.3	31.5	31.6	31.8	31.9	32.1
31	31.2	31.3	31.4	31.5	31.7	31.7	31.8	32.0	32.2	32.4	32.5	32.6	32.8	33.0	33.1
32	32.2	32.3	32.5	32.6	32.7	32.9	33.0	33.2	33.3	33.4	33.6	33.7	33.9	34.0	34.2
33	33.2	33.3	33.5	33.6	33.8	33.9	34.0	34.2	34.3	34.5	34.6	34.7	34.9	35.1	35.2
34	34.2	34.3	34.5	34.6	34.8	34.9	35.0	35.2	35.3	35.5	35.6	35.8	36.0	36.1	36.3
35	35.2	35.3	35.5	35.6	35.8	35.9	36.1	36.2	36.4	36.5	36.7	36.8	37.0	37.2	37.3

SOLIDS-NOT-FAT IN MILK CALCULATED BY BABCOCK'S FORMULA FROM QUEVENNE LACTOMETER READINGS AND PER CENT OF FAT *

Fat %	Quevenne Lactometer Reading at 15.5° C. (60° F.) †										
	25	26	27	28	29	30	31	32	33	34	35
0.0	6.25	6.50	6.75	7.00	7.25	7.50	7.75	8.00	8.25	8.50	8.75
0.1	6.37	6.62	6.87	7.12	7.37	7.62	7.87	8.12	8.37	8.62	8.87
0.2	6.49	6.74	6.99	7.24	7.49	7.74	7.99	8.24	8.49	8.74	8.99
0.3	6.61	6.86	7.11	7.36	7.61	7.86	8.11	8.36	8.61	8.86	9.11
0.4	6.73	6.98	7.23	7.48	7.73	7.98	8.23	8.48	8.73	8.99	9.23
0.5	6.85	7.10	7.35	7.60	7.85	8.10	8.35	8.60	8.85	9.10	9.35
0.6	6.97	7.22	7.47	7.72	7.97	8.22	8.47	8.72	8.97	9.22	9.47
0.7	7.09	7.34	7.59	7.84	8.09	8.34	8.59	8.84	9.09	9.34	9.59
0.8	7.21	7.46	7.71	7.96	8.21	8.46	8.71	8.96	9.21	9.46	9.71
0.9	7.33	7.58	7.83	8.08	8.33	8.58	8.83	9.08	9.33	9.58	9.83
1.0	7.45	7.70	7.95	8.20	8.45	8.70	8.95	9.20	9.45	9.70	9.95
1.1	7.57	7.82	8.07	8.32	8.57	8.82	9.07	9.32	9.57	9.82	10.07
1.2	7.69	7.94	8.19	8.44	8.69	8.94	9.19	9.44	9.69	9.94	10.19
1.3	7.81	8.06	8.31	8.56	8.81	9.06	9.31	9.56	9.81	10.06	10.31
1.4	7.93	8.18	8.43	8.68	8.93	9.18	9.43	9.68	9.93	10.18	10.43
1.5	8.05	8.30	8.55	8.80	9.05	9.30	9.55	9.80	10.05	10.30	10.55
1.6	8.17	8.42	8.67	8.92	9.17	9.42	9.67	9.82	10.17	10.42	10.67
1.7	8.29	8.54	8.79	9.04	9.29	9.54	9.79	10.04	10.29	10.54	10.79
1.8	8.41	8.66	8.91	9.16	9.41	9.66	9.91	10.16	10.41	10.66	10.91
1.9	8.53	8.78	9.03	9.28	9.53	9.78	10.03	10.28	10.55	10.78	11.04
2.0	8.65	8.90	9.15	9.40	9.65	9.90	10.15	10.40	10.66	10.91	11.16
2.1	8.77	9.02	9.27	9.52	9.77	10.02	10.27	10.52	10.78	11.03	11.28
2.2	8.89	9.14	9.39	9.64	9.89	10.14	10.39	10.64	10.90	11.15	11.40
2.3	9.01	9.26	9.51	9.76	10.01	10.26	10.51	10.76	11.02	11.27	11.52
2.4	9.13	9.38	9.63	9.88	10.13	10.38	10.63	10.88	11.14	11.39	11.64
2.5	9.25	9.50	9.75	10.00	10.25	10.50	10.75	11.00	11.26	11.51	11.76
2.6	9.37	9.62	9.87	10.12	10.37	10.62	10.87	11.12	11.38	11.63	11.88
2.7	9.49	9.74	9.99	10.24	10.49	10.74	10.99	11.24	11.50	11.75	12.00
2.8	9.61	9.86	10.11	10.36	10.61	10.86	11.11	11.37	11.62	11.87	12.12
2.9	9.73	9.98	10.23	10.48	10.73	10.98	11.23	11.49	11.74	11.99	12.24
3.0	9.85	10.10	10.35	10.60	10.85	11.10	11.36	11.61	11.86	12.11	12.36
3.1	9.97	10.22	10.47	10.72	10.97	11.23	11.48	11.73	11.98	12.23	12.48
3.2	10.09	10.34	10.59	10.84	11.09	11.35	11.60	11.85	12.10	12.35	12.61
3.3	10.21	10.46	10.71	10.96	11.22	11.47	11.72	11.97	12.22	12.48	12.73
3.4	10.33	10.58	10.83	11.09	11.34	11.59	11.84	12.09	12.34	12.60	12.85
3.5	10.45	10.70	10.95	11.21	11.46	11.71	11.96	12.21	12.46	12.72	12.97
3.6	10.57	10.82	11.08	11.33	11.58	11.83	12.08	12.33	12.58	12.84	13.09
3.7	10.79	10.94	11.20	11.45	11.70	11.95	12.20	12.45	12.70	12.96	13.21
3.8	10.81	11.06	11.32	11.57	11.82	12.07	12.32	12.57	12.82	13.08	13.33
3.9	10.93	11.18	11.44	11.69	11.94	12.19	12.44	12.69	12.94	13.20	13.45
4.0	11.05	11.30	11.56	11.81	12.06	12.31	12.56	12.81	13.06	13.32	13.57
4.1	11.17	11.42	11.68	11.93	12.18	12.43	12.68	12.93	13.18	13.44	13.69
4.2	11.29	11.54	11.80	12.05	12.30	12.55	12.80	13.05	13.31	13.56	13.82
4.3	11.41	11.66	11.92	12.17	12.42	12.67	12.92	13.18	13.43	13.68	13.94
4.4	11.53	11.78	12.04	12.29	12.54	12.79	13.04	13.30	13.55	13.80	14.06
4.5	11.65	11.90	12.16	12.41	12.66	12.91	13.16	13.42	13.67	13.92	14.18
4.6	11.78	12.03	12.28	12.53	12.78	13.03	13.28	13.54	13.79	14.04	14.30
4.7	11.90	12.15	12.40	12.65	12.90	13.15	13.40	13.66	13.91	14.16	14.42
4.8	12.02	12.27	12.52	12.77	13.02	13.27	13.52	13.78	14.03	14.28	14.54
4.9	12.14	12.39	12.64	12.89	13.14	13.39	13.64	13.90	14.15	14.40	14.66
5.0	12.26	12.51	12.76	13.01	13.26	13.51	13.76	14.02	14.27	14.52	14.78
5.1	12.38	12.63	12.88	13.13	13.38	13.63	13.89	14.14	14.39	14.64	14.90
5.2	12.50	12.75	13.00	13.25	13.50	13.75	14.01	14.26	14.51	14.76	15.02
5.3	12.62	12.87	13.12	13.37	13.62	13.87	14.13	14.38	14.63	14.88	15.14
5.4	12.74	12.99	13.24	13.49	13.71	14.00	14.25	14.50	14.76	15.01	15.26
5.5	12.86	13.11	13.36	13.61	13.86	14.12	14.37	14.62	14.88	15.13	15.38

* Abridged from *Mass. State Board Health Rept.* 1901, p. 445. † Prefix 1.0 if the lactometer scale shows specific gravity.

' C. (60° F.) or corrected to that temperature by means of Vieth's table above.

Derive the percentage of solids-not-fat from the table herewith or by the Babcock formula,[18] on which the table is based, as follows:

$$100g - gf \qquad - 1\Big) (100 - f)2.5$$

in which S is the solids-not-fat, g is the specific gravity, and f is the percentage of fat.

Protein

Kjeldahl Nitrogen Method. Pipet 5 ml. of the well-mixed sample into a Kjeldahl digestion flask and proceed as directed in Part I, C1*c*. At the same time that the portions are introduced into the digestion flask, pipet a 5-ml. portion into a dish or balanced watch glass and determine its weight. If a considerable number of determinations are made, calculation may be simplified by employing a pipet delivering 5 g. of milk of average specific gravity. The factor for conversion of nitrogen into protein is 6.38.

Sørensen-Pool Formol Volumetric Method.[19] The well-known Sørensen formol titration method for proteins and amino acids (titration of the carboxyl group after rendering the amino group amphoteric) has been adapted by Pool for the rapid determination of proteins in milk. The method is stated to yield results within 0.2% of those by the Kjeldahl method and is used by military pharmacists in the Dutch East Indies.

Reagent. *Formaldehyde, 40%.* Accurately neutralize to phenolphthalein with NaOH solution.

Process. Neutralize 100 ml. of milk to *phenolphthalein* with *0.25 N hydrochloric acid*, add 20 ml. of *neutral formaldehyde*, and titrate with standard *0.25 N sodium hydroxide solution*. The number of milliliters required is the formol number.

Calculation. Multiply the formol number by the factor 0.495 or, for greater convenience with little loss of accuracy, 0.5. The product is the per cent of protein.

Individual Proteins

Van Slyke Comprehensive Method.[20] Process. A and B are official A.O.A.C. methods.

A. Casein. Warm 10 g. of the sample diluted in a beaker to 100 ml. to 40 to 42°, add 1.5 ml. of *10% by weight acetic acid*, stir, and allow to stand for 3 to 5 minutes, maintaining the temperature of 40 to 42° until a flocculent precipitate separates from a clear supernatant liquid. Filter and wash with cold water 2 or 3 times, at first by decantation, then collect the entire precipitate on the filter and wash with 2 or 3 additional portions of cold water.

If the filtrate is not clear at any stage, return it to the filter, repeating if necessary. Transfer the filter containing the casein to a Kjeldahl flask, determine the nitrogen by one of the standard methods, and calculate the weight of casein, using the factor 6.38.

B. Albumin. To the filtrate from the casein precipitate, as above obtained, add *sodium hydroxide solution* to exact neutralization, then 0.3 ml. of *10% acetic acid* and boil until all the albumin is precipitated. Collect the precipitate on a filter, wash with hot water, determine nitrogen, and calculate the percentage of albumin, using the factor 6.38.

C. Caseoses. Heat the filtrate from the albumin to 70°, add 1 ml. of *50% sulfuric acid*, then *zinc sulfate* to the saturation point. Allow to stand at 70° until the caseoses separate and settle. After cooling, filter, and wash with *saturated zinc sulfate solution* slightly acidulated with *sulfuric acid*. Determine nitrogen in the precipitate and express results as caseose nitrogen.

D. Amino Compounds and Ammonia. To 50 g. of the milk contained in a 250-ml. volumetric flask, add 1 g. of *sodium chloride*, and, drop by drop, *12% tannin solution* until no more precipitate forms. Make up to the mark, shake, filter, and determine nitrogen as amino compounds and ammonia in 50 ml. (equivalent to 10 g. of the sample).

Ammonia. In another aliquot of the filtrate from the tannin precipitate, determine nitrogen as ammonia by distillation with *magnesium oxide* and titration. By difference obtain the nitrogen as amino compounds.

E. Peptones. To obtain the peptone nitrogen, subtract the sum of the nitrogen in the forms determined as above described from the total nitrogen.

Moir Comprehensive Method.[21] Casein is defined as the material precipitated from cow's milk at *p*H 4.6 by acetic acid buffered with sodium acetate. After precipitation of the casein, the albumin and globulin are determined together or separately, the latter by either of two methods.

Reagent. *Acid-Acetate Buffer.* Mix 90 ml. of 1.0 N acetic acid with 35 ml. of 1.0 N NaOH solution, thereby obtaining acid and sodium acetate concentrations of 0.44 N and 0.28 N respectively at *p*H 4.42.

Process. A. Casein. Pipet 10 ml. of milk into a tared covered beaker and quickly weigh. Dilute with about 50 ml. of warm (40 to 42°) water, at once add 1.5 ml. of *10% acetic acid*, stir gently by rotating a stirring rod four times in the beaker, and let stand for about 20 minutes, then add 4.5 ml. of *acid-acetate buffer*, gently stir, and let stand for at least 1 hour. Filter through a 9-cm. Whatman pleated paper, wash the precipitate with water three times by decantation, then twice more, breaking up the precipitate, transferring to the paper, and rinsing the rim. Carry out the filtration and washing without interruption to prevent drying out of the casein. Place 20 ml. of water in the beaker and pour gently down the sides the *sulfuric acid* for the Kjeldahl digestion, thus dissolving the adhering casein. Transfer the precipitate, paper, and the acid to a Kjeldahl flask and determine the nitrogen by the Kjeldahl-Arnold method.

B. Albumin Plus Globulin. To the filtrate from the casein, add *trichloracetic acid* to a concentration of about 4%. Heat for 30 minutes on a boiling water bath, cool, filter, wash with *1% trichloracetic acid solution*, and determine the nitrogen in the precipitate as above.

C. Globulin. (1) Neutralize the filtrate from the casein to *phenolphthalein* with *0.1 N sodium hydroxide solution* and saturate with a weighed amount of *anhydrous magnesium or sodium sulfate*, stirring at 20° on a water bath to hasten solution. After allowing to stand some time, filter and wash with *saturated sodium chloride solution* without interruption. If mechanical impurities are present, wash the globulin through the paper into the Kjeldahl flask with water or very *dilute sodium chloride solution*. Determine nitrogen as above.

(2) Neutralize 10 ml. of milk, mix with at least 90 ml. of *saturated sodium or magnesium sulfate solution*, and add sufficient extra *salt* to saturate 10 ml. of water. Collect the casein plus globulin precipitate on a filter, wash with *saturated sodium chloride solution*, and determine nitrogen as above. Deduct the casein nitrogen, thus obtaining the globulin nitrogen.

D. Albumin. Acidify the filtrate from either of the globulin methods with 3 ml. of *10% acetic acid*, heat on a boiling water bath at least 30 minutes, cool, filter off the precipitate, and wash with *saturated sodium chloride solution*. Transfer the filter and precipitate, also rinse the beaker, into a Kjeldahl flask with a little *sodium hydroxide solution*, and determine nitrogen by the Kjeldahl-Arnold method, heating gently during the early stages.

Casein

Van Slyke and Bosworth Acetic Acid Volumetric Method.[22] Although specially designed by the originators for the use of the dairyman with some experience in manipulation, but not provided with a chemical balance, the method is of real scientific merit. It may be applied to fresh milk, even if preserved with corrosive sublimate (1 : 1000 or 1500), but not to sour milk.

Reagents. *Phenolphthalein Solution.* Dissolve 1 g. of phenolphthalein in 100 ml. of 50% ethanol with alkali sufficient to give a very slight pink color.

Standard Alkali. Dilute 100 ml. of 1.0 *N* NaOH to 1260 ml.

Standard Acetic Acid. Dilute 100 ml. of normal acid to 1260 ml.

Process. *Neutralization.* Pipet 17.5 ml. (18 g.) of milk into a 200-ml. graduated flask, add 80 ml. of water and 1 ml. of *phenolphthalein solution*, then neutralize cautiously with *standard alkali*, shaking vigorously after each addition, until a faint, but distinct, color remains.

Casein Precipitation. To the neutralized solution at 16 to 26°, add *standard acetic acid* from a buret in 5-ml. portions up to 25 ml., then allow to rest for a short time. If sufficient acid has been added, the casein will separate promptly in large flakes and the supernatant liquid will be clear. If 25 ml. are not sufficient, add 1-ml. portions, shaking after each addition, until the casein separates promptly and completely. Record the number of milliliters of standard acid used.

Titration. Make up to the mark, shake, and filter through a dry paper into a dry flask. If the filtrate is not clear, repeat the determination, using more *acetic acid.* Titrate 100 ml. of the filtrate with the *standard alkali* to a faint, but distinct, pink color.

Calculation. Divide the number of milliliters of acid used by 2 and subtract from the quotient the number of milliliters of alkali required for the titration.

McDowall Acid-Acetate-Formol Volumetric Method.[23] Process. *Precipitation of Proteins.* Dilute 20 ml. of milk with 100 ml. of water at 42°. Precipitate the proteins with *sodium acetate in acetic acid* (see Moir Method above). Allow to stand 1 hour, then decant through a Gooch crucible with a removable bottom, keeping back the precipitate as completely as possible. Add 90 ml. of water to the residue in the beaker, mix, and after 5 minutes decant the liquid, repeating twice.

Titration. Transfer the asbestos mat and filter disk to the beaker containing the precipitate, add 11 ml. of *standard 0.1 N sodium hydroxide solution,* heat 5 minutes on a boiling water bath with occasional stirring, and neutralize to *phenolphthalein* with *0.2 N hydrochloric acid.* Add 4 ml. of *40% formaldehyde* and titrate with the *standard alkali* to the same end-point.

Calculation. The number of milliliters used in the last titration times 1.05 gives the per cent of casein in the milk.

Waterman Acid-Acetate Kjeldahl Method.[24] By using a fixed volume of acid-acetate buffer, Waterman (U. S. Department of Agriculture, Off. Exp. Sta., Washington) was able to dispense with the titration with acid. Perlman suggested diatomaceous earth as an aid to filtration. The present official A.O.A.C. Method is as follows.

Reagent. *Acid-Acetate Buffer.* Pipet 250 ml. of normal acetic acid and 125 ml. of normal CO_2-free NaOH solution into a 1-liter volumetric flask, and dilute to the mark with CO_2-free water.

Process. *Casein Precipitation.* Pipet 20 ml. of the milk into a 100-ml. volumetric flask, add 50 ml. of the *buffer*, mix, make up to the mark with water, and shake well. Heat in water at 50 to 60° for 15 minutes, and cool to room temperature, then add 0.5 g. of *celite analytical filter aid* and shake thor-

oughly. Filter through a pleated paper, avoiding evaporation.

Nitrogen Determination. Pipet 50 ml. of the clear filtrate into a Kjeldahl flask and determine the nitrogen. Determine nitrogen also in 10 ml. of the milk.

CALCULATION. Subtract the nitrogen in the filtrate from the total nitrogen and multiply the difference by 6.38, thus obtaining the casein in 10 ml. of the sample.

Report grams of casein per 100 ml. of milk. Divide the latter by the specific gravity of the milk to obtain the per cent by weight.

Brereton and Sharp Acetic Acid Refractometric Method for Skim Milk.[25] This method, devised at Cornell University, has the advantage that the normality of the alkali and the reading temperature may vary, provided both conditions are the same for unknown and blank.

PROCESS. *Acid Precipitation.* Deliver 25 g. of skim milk at 40 to 42°, from a pipet calibrated for a specific gravity of 1.036, into a beaker. Add with stirring 225 ml. of tap water at 40 to 42° containing 3.8 ml. of *10% acetic acid,* let stand for 3 minutes, then decant through a 12.5-cm. No. 4 Whatman filter paper. Wash the precipitate twice by decantation with 75-ml. portions of *0.02% acetic acid* (*p*H 4.6). Transfer the casein to the filter, then refilter the entire filtrate. When all the liquid has passed through, place the casein and filter in a 70-ml. test tube, 25 mm. in diameter, with a mark showing exactly 51.2 ml.

Alkali Treatment. Add 25 ml. of *0.2 N sodium hydroxide solution,* macerate the casein and filter with a rubber-tipped rod, rinse the rod with water, work out air bubbles, and dilute further to the mark. Since the filter occupies 1.2 ml., the solution of the casein measures 50 ml. and the alkali concentration is 0.1 *N*. Continue the maceration with the rod, stir occasionally for 30 minutes, and filter through a dry No. 3 Whatman filter paper.

Refractometer Reading. Determine the refraction at about 25° with a dipping refractometer.

Blank. Make a blank determination, beginning with the placing of the filter paper in the test tube, then proceed in other respects as described, taking care that the readings of blank and unknown are at the same temperature.

CALCULATION. Subtract the reading of the blank from that of the unknown and divide the difference by 2.37, thus obtaining the percentage of casein in the sample.

EXAMPLES. In skim milk the casein by the refractometric method ranged from 1.98 to 3.90%, Holstein and Jersey skim milk being the extremes, Guernsey and Ayrshire intermediate. Compared with the A.O.A.C. Method, the maximum error for 21 samples was 0.06%.

Hart Acetic Acid Centrifugal Method.[26] PROCESS. Measure 2 ml. of *chloroform,* 20 ml. of *0.25% acetic acid* (at 21°), and 5 ml. of milk (at 65 to 75°) into the special form of graduated tube designed for the method. Place the thumb over the mouth of the tube, invert, and shake well for 15 to 20 seconds. Within 10 minutes, whirl in the special hand centrifuge (the revolving wheel of which, with tubes extended, is 15 inches in diameter) for 7.5 to 8 minutes at the rate of 2000 r.p.m., using a metronome to be certain that the variation either side is not more than 50 revolutions. Remove from the centrifuge, place in an upright position, and after 10 minutes read the percentage of the casein column, which is between the chloroform and aqueous columns, on the scale.

The test may be carried out with fresh milk or composite samples a week old preserved with potassium bichromate according to directions.

NON-PROTEIN NITROGEN

Denis and Minot Comprehensive Method.[27] APPARATUS. *Colorimeter.*

Autoclave.

Van Slyke Micro Apparatus for Amino Nitrogen.

REAGENTS. *Acid Digestion Mixture:* 100 ml. of 85% H_3PO_4, 300 ml. of H_2SO_4, and 15 ml. of 10% $CuSO_4 \cdot 5H_2O$.

Phosphotungstic Acid Reagent.[28] Heat 10 g. of $Na_2WO_4 \cdot 2H_2O$, 80 ml. of 85% H_3PO_4, and 750 ml. of water for 2 hours under a reflux condenser, cool, and make up to 1 liter.

PROCESS. A. TOTAL NON-PROTEIN NITROGEN. *Protein, Fat, and Lactose Removal.* (*a*) *Cow's Milk.* Pipet 10 ml. of milk into a 100-ml. volumetric flask, add 50 ml. of water and 20 ml. of *10% copper sulfate solution* to which has been added a sufficient amount of *sulfuric acid* to make a 0.005 *N* solution, and shake. Place in a boiling water bath for 20 minutes, cool, make up the mark with distilled water, and filter through a dry paper. Place 75 ml. of the filtrate in a 100-ml. volumetric flask, add 1 ml. of *30% ammonia-free formaldehyde* and 20 ml. of *10% calcium oxide suspension*, allow to stand at room temperature for 30 minutes, make up to volume, and filter through a dry paper. The solution should be clear and colorless, free from lactose, and with only a trace of copper. Precipitate the excess of calcium oxalate with 0.8 g. of a powdered mixture of 5 parts of *oxalic acid* and 2 parts of *potassium oxalate*, centrifuge, and add a crystal of the *oxalate* to verify the complete precipitation.

Pipet 20 ml. (= 1.5 ml. of milk) into a 20 x 200 mm. Pyrex test tube, add a glass bead and 1 ml. of *acid digestion mixture*, evaporate nearly to dryness with a flame or over a boiling *saturated calcium chloride solution*. When nearly dry, heat with a micro burner for 1 minute after the contents become colorless, keeping the tube covered with a watch glass or an inverted Erlenmeyer flask, the total time of heating being limited to 3 minutes. Cool, add water, and determine ammonia colorimetrically by (1) direct Nesslerization according to the Folin and Denis method,[29] (2) titration with *0.02 N sodium hydroxide solution* and *methyl red indicator* after aeration according to the Folin and Farmer method,[30] or (3) distillation according to the Bock and Benedict method.[31]

(*b*) *Human Milk.* To 10 ml. of milk in a 100-ml. volumetric flask, add 20 ml. of *10% copper sulfate solution* (omitting the sulfuric acid used for cow's milk), 30 to 50 ml. of water, and 1.5 ml. of *10% disodium phosphate solution*. Heat 20 to 30 minutes in a boiling water bath, cool, make up to volume with distilled water, and filter through a dry paper. Then proceed as with cow's milk.

B. UREA. Employ the Marshall urease method[32] as follows: Allow 5 ml. of cow's milk or 3 ml. of human milk to stand for 30 minutes in contact with 2 ml. of *jack bean extract*, add 1 g. of *solid potassium carbonate* and 1 to 2 drops of *kerosene*, and aerate for 15 minutes. Collect the ammonia in a 100-ml. volumetric flask containing 2 ml. of *0.1 N hydrochloric acid* and 25 ml. of water. Make up with water to 60 to 70 ml., add 15 ml. of *Nessler solution* and water to the mark, then read in a colorimeter with suitable standard which for normal milk may be one containing 0.5 mg. of *ammonia nitrogen* in a volume of 100 ml.

C. CREATININE AND CREATINE. (*a*) *Denis Method.* Proceed according to the Denis method[33] as follows. Treat 10 ml. of milk in a 50-ml. volumetric flask with an equal volume of water and 3 ml. of freshly prepared *20% m-phosphoric acid solution*, allow to stand 1 hour, fill to the mark with distilled water, mix, and filter through a dry paper.

1. *Preformed Creatinine.* Place 10 ml. of the above filtrate in a 25-ml. volumetric flask containing 10 ml. of *1.2% picric acid solution* and 1.5 ml. of *10% sodium hydroxide solution*. After allowing to stand at room temperature for 10 minutes, dilute to the mark and read in a colorimeter against a *standard* which has been standing the same length of time. A suitable standard for cow's

milk contains 10 ml. of *0.9% lactose solution*, 0.025 mg. of *creatinine*, 10 ml. of *picric acid solution*, and 1 ml. of *10% sodium hydroxide solution* made up to a volume of 25 ml. For human milk, substitute *1.4% lactose* for the 0.9% directed for cow's milk.

2. *Total Creatinine* (*Creatine plus Creatinine*). Autoclave 10 ml. of the *m*-phosphoric acid filtrate at 120° for 30 minutes. Cool, treat with *picric acid solution* and *sodium hydroxide solution* as described above, allow to stand for 10 minutes, and dilute to 50 ml. A suitable standard contains 0.05 mg. of *creatinine* in a final volume of 50 ml. with *lactose*, etc., added as above.

(*b*) *Van Slyke Method*. Precipitate lactose, etc., as follows. To 10 ml. of milk in a 50-ml. volumetric flask, add 5 ml. of *20% copper sulfate solution* and 15 ml. of *10% calcium oxide suspension*, add water to the mark, allow to stand for 30 minutes, centrifuge, and filter the supernatant liquid through a small dry paper.

1. *Preformed Creatinine*. To 10 ml. of the clear colorless filtrate in a 25-ml. volumetric flask, add 2 drops of *1.0 N hydrochloric acid*, 10 ml. of *saturated picric acid solution*, 1 ml. of *10% sodium hydroxide solution*, allow to stand 10 minutes, make up to the mark, and read against the following *standard:* 0.025 mg. of *creatinine* in 10 ml. of water, 10 ml. of *picric acid*, and 1 ml. of *10% sodium hydroxide solution*, made up to 25 ml.

2. *Total Creatinine*. Treat 10 ml. of the filtrate in a 50-ml. volumetric flask with 2 drops of *1.0 N hydrochloric acid* and 10 ml. of *picric acid solution*, and autoclave 30 minutes at 120°. Cool, treat with 1 ml. of *10% sodium hydroxide solution*, allow to stand 30 minutes, make up to volume, and read in the colorimeter against a *standard* containing 0.05 mg. of *creatinine*, 10 ml. of *picric acid solution*, and 1 ml. of *10% sodium hydroxide solution* in 50 ml. Centrifuge off the precipitated calcium hydroxide before reading in the colorimeter.

D. Amino Compounds. Determine by the Van Slyke nitrous acid method,[34] using micro apparatus after the protein precipitation as follows.

(*a*) *Protein Precipitation for Cow's Milk*. Pipet 20 ml. of milk into a 200-ml. volumetric flask, add 40 ml. of *0.01 N acetic acid*, 10 ml. of *5% copper acetate solution*, and 50 to 60 ml. of water, then place in a boiling water bath for 20 to 30 minutes. Add 1 ml. of *15% potassium oxalate solution*, cool, make up to the mark, and filter through a dry paper. Add to the filtrate 0.5 g. of *powdered potassium oxalate*, shake 1 to 2 minutes, and remove the precipitate by centrifuging.

Amino Nitrogen. Evaporate 50 ml. of the above clear filtrate to 1 to 2 ml. and determine the amino nitrogen with the Van Slyke micro apparatus.

(*b*) *Protein Precipitation for Human Milk*. Place 20 ml. of breast milk in a 200-ml. volumetric flask and treat with 10 ml. of *5% copper acetate solution*, 1 ml. of *10% disodium phosphate solution*, and 60 to 80 ml. of water. Heat 20 to 30 minutes on a boiling water bath, cool, make up to volume with water, mix, and filter through a dry paper. Evaporate 50 ml. of the filtrate to about 2 ml. and use for the determination of amino nitrogen in the usual manner. Addition of 2 to 3 drops of *glacial acetic acid* facilitates the transfer to the deaminizing bulb.

E. Uric Acid. Employ the Morris alkaline zinc acetate precipitation method [35] as follows. To 20 ml. of milk in a 100-ml. volumetric flask, add 40 ml. of *0.01 N acetic acid* and 1 ml. of *10% zinc acetate solution*, then heat in a boiling water bath for 15 to 20 minutes. Cool, make up to volume, and filter through a dry paper. The filtration should be rapid and the filtrate absolutely clear.

To 75 ml. of the filtrate, add 3 ml. of *10% zinc acetate solution* and 4 ml. of *20% sodium carbonate solution*, allow to stand at room temperature for 10 minutes, and centrifuge. Wash the gelatinous precipitate of zinc car-

bonate once with cold water and centrifuge a second time. Pour off the wash water, add to the residue 2 ml. of *50% acetic acid*, transfer with 25 to 35 ml. of water to an Erlenmeyer flask, and heat to boiling. Precipitate the zinc with *hydrogen sulfide gas*, filter, and wash the precipitate twice with hot water. Boil the filtrate and washings rapidly until free from hydrogen sulfide and reduced in volume to about 15 ml., cool, add 1 ml. of *phosphotungstic acid reagent* and 10 ml. of *20% sodium carbonate solution*, and make up to 25 to 50 ml. according to the degree of color. Read in a colorimeter against a *standard* such as 0.5 mg. of *uric acid* in a volume of 100 ml.

Urea

Perkins Urease Volumetric Method.[36] The method originated at Ohio Agricultural Experiment Station.

Reagent. *Jack Bean Meal Extract, 5%* in 25% ethanol.

Process. *Solution.* Prepare as directed for the Perkins Modification of the Magnesium Oxide Method, under Ammonia below.

Urease Digestion. To 20 ml. of the solution (equal to 4 ml. of milk) or other convenient volume, add half the volume of *5% jack bean meal extract*, dilute to 200 ml., and digest for 2 hours at 40° or allow to stand at room temperature overnight.

Distillation and Titration. Add 1 g. of *magnesium oxide* and proceed as directed under Ammonia below.

Calculation. Deduct the amount of preformed ammonia nitrogen and convert the remaining nitrogen into urea by the factor 2.214.

Ammonia

Perkins Distillation Method.[37] In the determination of ammonia by the magnesium oxide method in feeding experiments at the Ohio Agricultural Experiment Station, the following technique is employed.

Process. *Defecation.* Treat 100 ml. of milk with 20 g. of *anhydrous magnesium sulfate*, add *85 to 95% ethanol*, with one intermediate shaking, to a final volume of 500 ml. Allow to stand a short time and filter, pressing the paper, wrapped with a cloth, in the hand to secure a volume of about 430 ml.

Distillation and Titration. Distil an aliquot of 200 ml. with 0.5 to 1.0 g. of *magnesium oxide* into *standard 0.00714 N sulfuric acid*, then titrate back with *standard 0.00714 N ammonium hydroxide*, using very dilute and carefully neutralized *methyl red indicator*.

Calculation. Use the formula: 1 ml. of the standard acid = 0.1 mg. of ammonia nitrogen. Correct for the ammonia nitrogen in the reagents as obtained in a blank determination.

See also Urea above.

Nitric Acid

The presence of nitrates in varying amounts in well water and its absence in milk as drawn from the udder serve as a basis for detecting watering. In court cases the results of quantitative determinations made on the dairyman's milk and the water of his barn well have been used to calculate the percentage of water added.

Soxhlet Diphenylamine Test. The blue color formed by the action of *diphenylamine* in acid solution on a solution of nitrates, a reaction long used in sanitary analysis, was applied by Soxhlet to the calcium chloride serum of milk.

I. Möslinger Modification.[38] Reagent. *Diphenylamine Reagent* (Soxhlet). Dissolve 20 mg. of diphenylamine in 20 ml. of 1 + 3 H_2SO_4 and dilute to 100 ml. with 1 + 3 H_2SO_4.

Process. *Preparation of Serum.* Add to 100 ml. of milk 1.5 ml. of *20% calcium chloride solution*, heat to boiling, and filter.

Color Formation. Place 2 ml. of *diphenyl amine reagent* in a small white porcelain dish

and add to the middle of the liquid dropwise 0.5 ml. of the serum. Allow to stand 2 to 3 minutes, then move the dish back and forth and again allow to stand undisturbed. Repeat several times and note the formation of blue streaks and finally a more or less intense blue color indicative of nitric acid.

II. Tillmans and Splittgerber Modification.[39] Reagents. *Diphenylamine Reagent.* Prepare as directed above.

Potassium Nitrate Solution. Dissolve 0.1871 g. of KNO_3 in water and dilute to 1 liter.

Process. *Preparation of Serum.* Place 25 ml. of milk in a 50-ml. glass-stoppered cylinder, add 25 ml. of a mixture of equal parts of *5% mercuric chloride solution* and *2% hydrochloric acid*, and shake for a moment. Filter on a folded paper.

Color Formation. To 4 ml. of the *diphenylamine reagent* in a test tube, add 1 ml. of the serum, shake, and cool under the faucet. Allow to stand for 1 hour, shaking occasionally. Milk containing water contaminated with nitrates produces a blue color, nitrate-free milk a yellow or rose tint.

Quantitative Determination. Pipet into each of a series of 100-ml. volumetric flasks 0.40, 0.80, 1.20, 1.60, and 2.00 ml. of *potassium nitrate solution,* add 2 ml. of cold *saturated sodium chloride solution* and 10 ml. of *glacial acetic acid,* and fill to the mark. The solutions correspond to 1, 2, 3, 4, and 5 mg. of N_2O_5 in 1 liter of milk and retain their strength indefinitely. Carry along tests on these solutions with the test on the sample and match the colors.

III. Lerrigo Modification.[40] Reagents. *Mercury Reagent.* Prepare an aqueous solution containing 20% $HgCl_2$, 5% NH_4Cl, and 20% (by volume) HCl.

Diphenylamine Reagent (Elsdon and Sutcliffe). Dissolve 0.085 g. of diphenylamine in 50 ml. of water and add gradually 450 ml. of H_2SO_4.

Process. Shake at intervals for 2 minutes in a test tube, previously washed with milk, a mixture of 4 or 5 ml. of milk and 6 to 7 drops of *mercury reagent.* Filter through a 9-cm. paper, washed with water until free from nitrates, into a 15 x 1 cm. test tube containing about 2 ml. of *diphenylamine reagent,* which has been introduced without unduly wetting the sides of the tube. In filtering, hold the test tube obliquely so that the filtered serum flows onto the surface of the reagent. After collecting about 1 ml. of serum, remove the funnel, hold the test tube erect, and examine against a white glazed surface.

Interpretation. *Normal milk:* demarcation between the serum and reagent colorless; gentle heating at this point produces a faint yellow color which changes to dark brown on charring. *Milk plus large amounts of nitrate:* surface of contact a dark blue layer which spreads upward on gentle shaking. *Milk plus small amounts of nitrate* (0.1 part nitric nitrogen per 100,000 or less): at first like normal milk, but on gentle shaking a blue color appears at the bottom of the serum and immediately beneath there soon appears the yellow color characteristic of normal milk.

Fat

Dry Extraction Gravimetric Methods. Since the adoption of the Babcock centrifugal method, gravimetric methods have not been regarded so essential in practical work as formerly; nevertheless it must not be forgotten that the ether-extraction gravimetric method was the standard for obtaining results with which all rapid methods are compared while on probation. The gravimetric method, theoretically at least, is exact in certain details in which all methods that measure the portion of the sample and the separated fat, assuming an average specific gravity for both, are only approximate.

Dish Method. Although well suited for the determination of solids, a portion of the sample weighed into a dish without an ab-

sorbent and dried does not permit satisfactory extraction of the fat by solvents as some have recommended.

Asbestos Method. After drying in a tinned lead dish or perforated cylinder with asbestos as described under Solids above, complete the extraction of the fat with *ether*. This is readily accomplished in about 4 hours, the Johnson extractor (Fig. 159) being used.

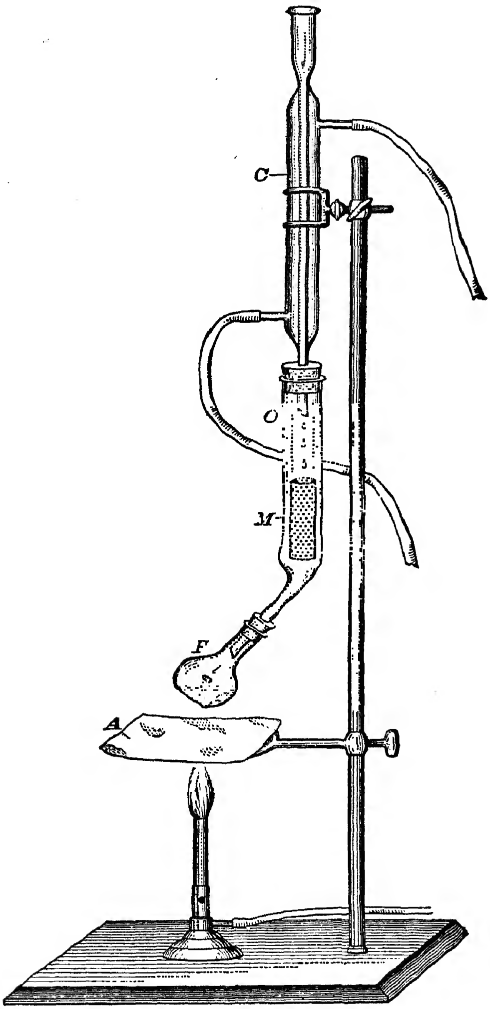


FIG. 159. Johnson Extractor with Perforated Cylinder.

Shred and crumple the lead dish so that it fits into the inner tube of the extractor, but dispense with the inner tube if the drying was carried out in a perforated cylinder. Proceed otherwise as in the extraction of powders.

The perforated cylinder with its non-fatty residue may be dried after extraction in the boiling-water oven for the determination of solids-not-fat or this may be determined by subtracting the result on fat from that on solids.

Sand Method. After determination of solids (see above), grind the sand impregnated with dried milk with a pestle, carefully avoiding loss, completely transfer to the inner tube of the Johnson extractor, rinse the dish with *ether* (or in case a tinned lead bottle cap was used place this on top of sand), and extract in the usual manner.

Paper Coil Method. Proceed with the dried coil in the same manner as with the perforated cylinder. See Solids above.

Make a blank determination on the coil and introduce the correction.

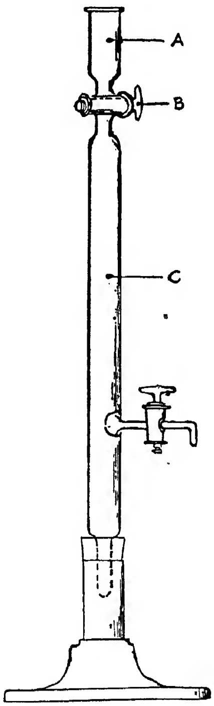
Wet Extraction Gravimetric Methods. In these methods the fat is shaken out with ether in connection with other reagents, the ether evaporated, and the fat weighed.

Röse-Gottlieb-Patrick Ammonia-Ethanol-Ether Gravimetric Method.[41] This method, in the experience of the writers, is less suited for milk than for sweetened condensed milk and ice cream which contain sucrose that interferes with ether extraction by the asbestos or sand methods employing the Johnson or similar extractor. The last two methods, applied to milk, involve simple manipulation and consume only about 10 ml. of ether, whereas the Röse-Gottlieb-Patrick method requires more than 40 ml. of ether, as well as

about 40 ml. of naphtha and 10 ml. of ethanol; furthermore the manipulation is more complicated.

APPARATUS. A *Röhrig Tube* (Fig. 160), as modified by Sullens and Rankin,[42] which differs from the original (Fig. 161) in that the cock and the reservoir above it replace the stopper. A cylinder 2 cm. in diameter and 40 cm. high, provided with a narrow siphon or tubes similar to those of a wash bottle, but with the longer tube narrowed at the bottom, is satisfactory for occasional determinations.

PROCESS. *First Extraction.* Pipet 10 ml. of a solution of 40 g. of the sample made up to 100 ml. into a modified Röhrig tube or cylinder, add 0.5 ml. of water, 1.25 ml. of



Courtesy of Ind. Eng. Chem., Anal. Ed. 1940, 21, 291

FIG. 160. Sullens and Rankin Modification of Röhrig Tube.

FIG. 161. Röhrig Tube, Original Form.

ammonium hydroxide, and mix well. If the sample is sour, add a total of 2 ml. of the *alkali*, then add 10 ml. of *ethanol* and shake again. Add 25 ml. of *washed ether* and 25 ml. of *naphtha redistilled below* 60°, shaking vigorously for 30 seconds before the next addition. After allowing to stand 20 minutes (or longer if the upper liquid is not clear and entirely separated), draw or siphon off the upper layer down to the last milliliter in the cylinder onto a very small filter and collect the filtrate in a weighed flask.

Second Extraction. Repeat the extraction, using only 15 ml. of each *solvent* and collecting the filtrate in the same flask.

Third Extraction. This is required if the separation after the first two is not sharp. Wash the paper with a few milliliters of a mixture of equal volumes of the two *solvents*.

Slowly evaporate the combined filtrates in a tared dish, dry in a boiling water oven for 1-hour periods until constant weight is reached.

After weighing, dissolve the fat in *naphtha*. If a residue is left in the flask, wash with

naphtha, dry, weigh, and correct the weight of the impure fat for this residue, also for the residue left on conducting a blank determination.

Schmidt-Werner Acid-Ether Gravimetric Method.[43] This method differs from the Röse-Gottlieb-Patrick method chiefly in that hydrochloric acid and purified ether replace ammonia and a mixture of ether and naphtha. In other respects the two methods are similar.

PROCESS. Pipet 9.8 ml. (equivalent to 10 g.) of the milk into a 75-ml. test tube, add 10 ml. of *hydrochloric acid*, shake, boil for 2 minutes or until the liquid is dark brown, and cool by immersion in cold water. Add to the mixture 30 ml. of *washed ether*, introduce a double-bored stopper with tubes as in a wash bottle, but with the lower end of the longer tube recurved, shake well, allow to settle, and carefully blow over the ether layer into a weighed dish. Repeat the extraction twice. Evaporate the ether, dry, and weigh.

Hydrometric Methods. **Babcock Formula Method.** A method involving calculation of the percentage of solids from the percentage of fat and the hydrometer (lactometer) reading is considered above (Babcock Formula Method). The reverse procedure of determining the fat from the specific gravity and per cent of solids may be carried out in conjunction with the table.

Soxhlet Alkali-Ether Method.[44] Before the introduction of the centrifugal methods, the Soxhlet method was highly regarded on the continent and still is described in German works. It is, however, cumbersome, expensive because of the amount of milk and ether required, and in the writers' experience far from accurate.

A mixture of the milk, *potash lye*, and *ether* is shaken in a bottle, allowed to stand, and the ether layer is pumped by a rubber bulb into a jacketed tube where the specific gravity is taken with a special hydrometer. The percentage of fat is found in a table.

Refractometric Methods. **Wollny Refractometric Method.**[45] The Zeiss instrument (Wollny refractometer) differs from the butyrorefractometer only in the arbitrary scale showing readings from 0 to 100 corresponding to refractive indices of from 1.3332 to 1.4220. Other apparatus is a multiple shaker, a centrifuge, and special glassware.

As in the Soxhlet hydrometric method, the fat is separated as an ether solution. The preliminary step is precipitation of the casein by *acetic acid*, after which the liquid is mixed with an *alkaline solution* containing *glycerin* and *copper hydroxide* and shaken with *ether*. The percentage of fat is determined either directly in the Wollny refractometer or by calculation in a butyrorefractometer or Abbé refractometer.

Butyrometric Methods. Numerous methods, depending on the measurement of the height of a cream, fat, or fatty acid column, have been devised with the special view of determining the butter-making value of milk as well as aiding in milk inspection.

Creamometer. The oldest and simplest of these tests consists in measuring the height of the cream column which separates from a given amount of milk in a specially graduated cylinder known as the creamometer. Unfortunately, even when the time and temperature of separation are carefully regulated, the fat content of the cream column is not the same for all samples, hence its height is not proportional to the fat content of the milk and the method is not reliable.

Fjord's Tester differs from the creamometer in that the cream is separated in small tubes by centrifugal force, but it is open to the same criticism as the creamometer.

Oil Test Churn. Although designed for cream, the apparatus is of interest in the development of milk methods. It consists of an aggregation of tubes serving as miniature churns in which the fat, after separation, is melted and the length of the liquid fat column is measured.

De Laval Lactocrite. This was the first really practicable tester of a number employing acids, alkalies, or other chemicals for dissolving the casein and other non-fatty substances. Centrifugal force was used to aid in separating the fat column.

In the United States, tests were devised by **Short, Failyer and Willard, Parsons, Cochran,** and **Patrick** in rapid succession, but none of these employed centrifugal force.

Short Method.[46] The fatty acids were separated and measured in a bottle, later employed

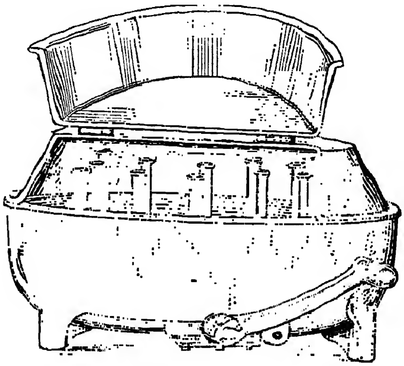


FIG. 162. Babcock Twelve-Bottle Centrifuge.

with modification by Babcock. In later methods the simpler procedure of separating the fat itself was found more practicable.

The **Babcock Test**[47] was the culmination of the series and soon was adopted throughout the United States. The **Leffmann and Beam Method,**[48] often known as the Beimling test, after the inventor of a centrifuge for the method, was described the same year, and during the succeeding years a number of European modifications, such as **Stokes Test, De Laval Test, Sin-Acid Test,** and **Gerber Test,** were placed on the market.

Sulfuric acid is used as a reagent in most of these methods either alone (Babcock, De Laval) or in conjunction with *amyl alcohol* (Stokes, Gerber), or *hydrochloric acid* and *amyl alcohol* (Leffmann and Beam); in the sin-acid method, however, an *alkaline mixture containing Rochelle salt, sodium sulfate, and sodium hydroxide,* in conjunction with *isobutanol,* is employed.

Babcock Sulfuric Acid Centrifugal Method.[47] Commonly known as the Babcock test, this method, more than any other, has served to place the American dairy industry on a chemical basis. Dr. Babcock was originally at the New York, and later at the Wisconsin, Experiment Station.

APPARATUS. A full set of standardized apparatus may be obtained from dealers in laboratory apparatus or in dairy supplies.

Centrifuge. A laboratory centrifuge with

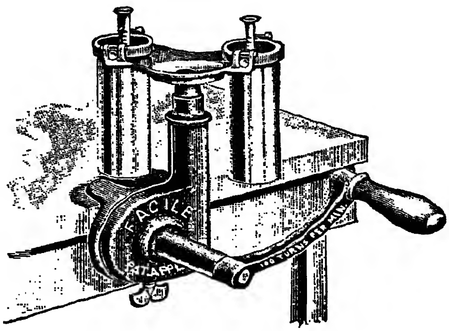


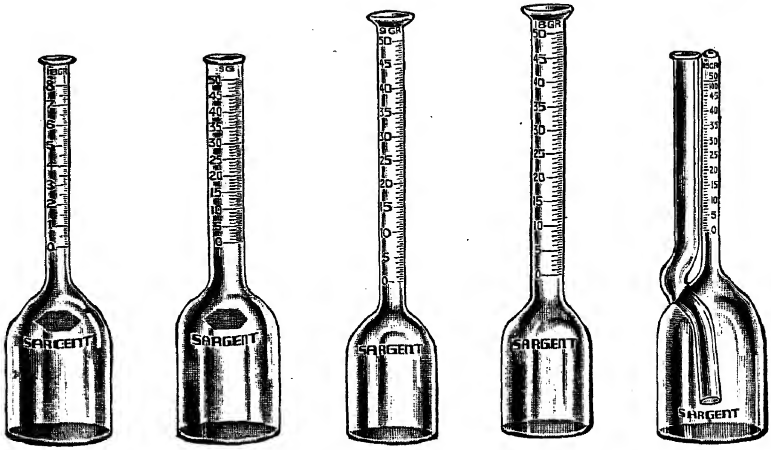
FIG. 163. Babcock Two-Bottle Centrifuge.

pockets for the test bottles is suitable. Special hand or power centrifuges, with a capacity from 2 test bottles upward, are made for dairy laboratories (Figs. 162 and 163). The steam turbine form is recommended because the steam serves to keep the bottles warm, as well as to supply power. Following are the diameters of centrifuges, recalculated to centimeters from inches, and the requisite number of revolutions per minute, as given by Farrington and Woll:[49] 25, 1074; 30, 980; 35, 909; 40, 848; 45, 800; 50, 759; 55, 724; 60, 693.

Test Bottles. The specifications of the Association of Official Agricultural Chemists and the American Public Health Association for milk bottles, three forms of cream bottles, and skim milk bottles are shown in the legend of Fig. 164. The milk test bottle, as devised

by Babcock, was graduated up to 10% and the cream bottle, devised by Winton, up to 30%; the former, however, has been reduced to 8% and the latter extended to 50%.

Hortvet's Special Calipers (Fig. 167) is a device designed to secure rapid and accurate readings. The knobs at the right adjust the pointers to the top and bottom meniscus, then, by turning the lower knob, the lower pointer is moved to 0 and the upper pointer shows the percentage.



I II III IV V

Courtesy of E. H. Sargent & Co., Chicago

FIG. 164. Babcock Milk Test Bottles: I Milk, 6 inch, 18 gram, 8%; II Cream, 6 inch, 9 gram, 50%; III Cream, 9 inch, 9 gram, 50%; IV Cream, 9 inch, 18 gram, 50%; V Skim Milk or Buttermilk, 6 inch, 18 gram, 0.50%.

Pipets. The pipet for milk delivers 17.6 ml. (Fig. 165) which is equivalent to 18 g. of milk of average composition. An 18-ml. pipet delivers about 18 g. of thin cream, but for accurate results the charge of either 18 g. or 9 g. should be weighed on the chemical balance or on a special scale accurate to 30 mg.

Acid Measure (Fig. 166). This may be a 17.5-ml. cylinder or an automatic pipet connected with a supply bottle.

Fat Column Calipers. Although, as in using a buret, the top and bottom of the fat column may be read and the percentage of fat obtained by subtraction, it is convenient to apply the points of calipers first to the top and bottom meniscus and then, with one point at 0 and the other farther along on the scale, read the percentage directly.

REAGENT. *Commercial Sulfuric Acid* (sp.gr. 1.82 to 1.83).

PROCESS. *Acid Treatment.* Rinse the 17.6-ml. pipet by shaking with a small amount of the well-mixed sample at moderate room temperature, then fill to the mark and transfer to the milk test bottle, blowing out the drop formed after draining for a few seconds, and finally add 17.5 ml. of *sulfuric acid* at moderate room temperature, while turning the bottle to rinse down the neck.

Mixing. Mix vigorously with a rotary motion, taking special care to hold the neck with the fingers at such an angle that any of the acid liquid that might escape will not reach the face. The acid acting at the high tem-

perature developed by mixing with the aqueous liquid dissolves the lumps of curd first formed and darkens the color.

Too high temperature or too strong acid causes a blackening of the fat; too low temperature or too weak acid results in imperfect

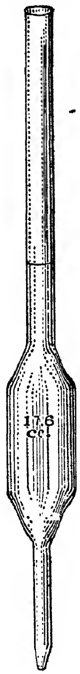


FIG. 165. Babcock Milk Pipet.

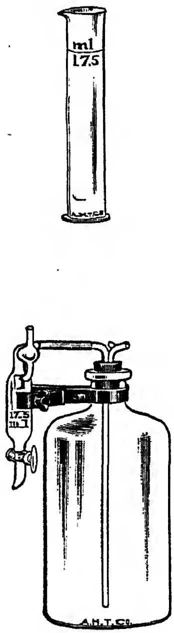


FIG. 166. Babcock Acid Measures: Top, Cylinder; Bottom, Special Form Attached to Acid Bottle (*Arthur H. Thomas Company, Philadelphia*).

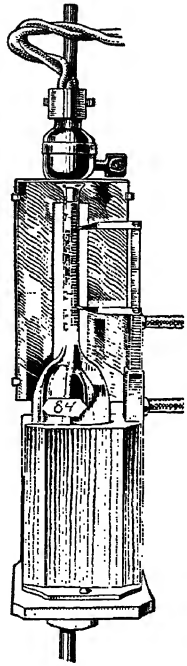


FIG. 167. Hortvet Fat Calipers.

solution of the curd and contamination later of the separated fat column.

Whirling. Arrange the bottles in the centrifuge symmetrically so as to obviate excessive vibration, using dummies if necessary. Whirl for 5 minutes at the proper speed, keeping the temperature within the centrifuge at about 60°. Fill to the neck with boiling water, whirl for 2 minutes, then fill nearly to the top of the graduation and whirl a third time. Some operators add water only once, but a clearer reading is usually secured by the double addition.

Reading. Remove the test bottles to a bath kept at 60° and read the percentage of fat at about that temperature. If the interior of the centrifuge is properly heated, the bottles may be removed one by one and read directly.

Gerber Sulfuric Acid-Amyl Alcohol Centrifugal Method.[50] This method is to Germany what the Babcock test is to the United States.

APPARATUS. *The Test Bottle* (butyrometer) differs from the Babcock form chiefly in that it is permanently closed at the top of the

graduated stem and has an opening at the constricted lower end of the bulb for filling which is closed with a rubber stopper during the test. Modified forms are designed for cream and skim milk.

Pipet: 11 ml. for milk, 3 ml. for cream, and 1 ml. for amyl alcohol; also a safety 10 ml. for acid.

Centrifuge.

REAGENTS. *Sulfuric Acid* (sp.gr. 1.815 to 1.825).

Amyl Alcohol, free from impurities yielding a measurable amount of fat-like substances in the process.

PROCESS. Introduce 10 ml. of *sulfuric acid* into the graduated bottle and allow 11 ml. of the sample to flow from a pipet down the sides so as to form a layer on the top of the acid. In like manner, add 1 ml. of *colorless amyl alcohol,* close with a rubber stopper, and shake vigorously with an up-and-down motion until the flocks of casein have dissolved completely. Heat in a water bath at 60 to 70° for 5 to 15 minutes and whirl 3 to 5 minutes in the centrifuge at the rate of 800 to 1000 r.p.m. Place in water at 60 to 70° and after some minutes read the volume of the fat which is brought into the neck by adjustment of the rubber stopper.

Make blank tests to determine whether the amyl alcohol contains matters which would be read as fat, also compare results from time to time with those by a standard method. The fat should not be pink, should be free from flocks, and should form a sharp line of demarcation from the liquid beneath.

LECITHIN

Nerking and Haensel Phosphoric Acid Gravimetric Method.[51] PROCESS. To 100 ml. of milk, add 200 ml. of *ethanol* with stirring. Filter and extract the precipitate and filter in a continuous extractor for 30 hours with *chloroform.* Evaporate at 50 to 60° the filtrate from the ethanol precipitate, add to the residue the chloroform solution obtained by the extraction of the ethanol precipitate, and evaporate to dryness in a platinum dish; add a small amount of *sodium carbonate* and *potassium nitrate,* ignite, and determine phosphoric acid by the molybdic-magnesium pyrophosphate method.

CALCULATION. The weight of magnesium pyrophosphate $\times$ 7.27 = the weight of lecithin of arbitrary molecular weight.

EXAMPLES. Mohr, Brockmann, and Muller[52] employed the Brodnick-Pittard method, details of which are not available, but they call attention to the uncertain composition of milk lecithin. They report the following figures: skim milk 0.0155, whole milk 0.0370, cream 0.1685, butter 0.2060, and whey 0.007%.

LACTIC ACID

von Fürth and Charnass Acetaldehyde Distillation Iodometric Method.[53] The oxidation of lactic acid by potassium permanganate, the determination of the acetaldehyde thus formed by distillation into bisulfite, and the iodometric titration as originated by Ripper[54] for aldehydes in general are the salient features of this method as developed at the University of Vienna.

I. Clausen Aeration Modification.[55] Devised originally (Washington University Medical School, St. Louis) for small amounts of lactic acid in urine and blood, the method may be adapted for milk, meat, and wine analysis.

APPARATUS. A *Train* consisting of (1) a Pyrex *reaction test tube* (20 x 200 mm.), provided with an inlet tube, (2) a 400-mm. *air condenser*, and (3) two *receiving tubes* arranged for absorption.

REAGENTS. *Bisulfite Solution,* 0.02 *N.* Pass SO_2 gas into a saturated solution of Na_2CO_3 until the color becomes pale greenish yellow and the odor of SO_2 is distinct.

Iodine Solution. Dilute a stock 0.1 *N* solution to 0.01 or 0.001 *N* containing 2% of KI.

Standardize against 0.1 or 0.005 *N* :
$5H_2O$ containing a little NaOH solution, and this in turn against standard 0.1 *N* KIO_3HIO_3 solution (3.2496 g. per liter).

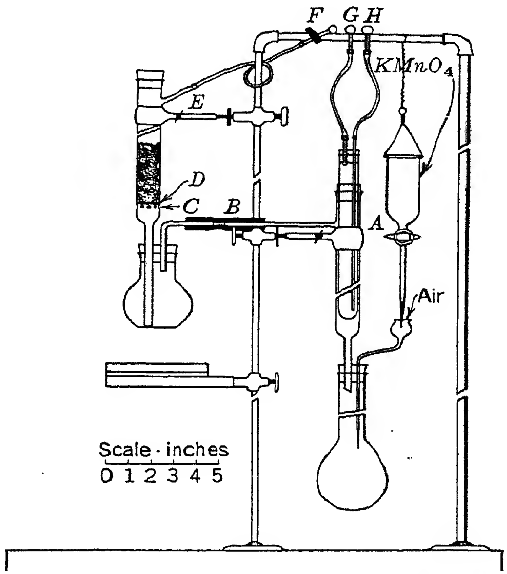
PROCESS. *Formation of Acetaldehyde and Aeration.* The micro adaptation of the von Fürth and Charnass procedure is carried out as follows. To the reaction test tube, containing a measured portion of the liquid sample or an extract of a tissue, add 5 to 10 ml. of *1% sulfuric acid.* Connect with the condenser, which previously has been connected with the two receiving tubes containing 20 ml. each of *0.02 N bisulfite solution.* While passing a fairly rapid air current through the train, add *0.005 N potassium permanganate solution* through the inlet tube dropwise and only as rapidly as decolorization is effected, then add water to the original volume. After 30 minutes when the permanganate no longer fades, run the air current through the tubes for 10 minutes longer, then transfer the contents of the receivers to a flask.

If the amount of lactic acid exceeds 10 mg., proceed as directed by Meissner and Schneyer, namely, add 5 to 10 ml. of *50% sulfuric acid* and allow to react at 140° for 1 hour while passing a rapid air current through the train.

Titration. Add to the solution containing acetaldehyde a considerable excess of *0.02 N bisulfite solution,* which does not need to be measured. After 10 minutes, add *0.1 N iodine solution* and *starch indicator* until a definite blue color is formed, then add *saturated sodium bicarbonate solution* to discharge the blue color. Finally titrate the bound sulfite with standard *0.01 or 0.001 N iodine solution.*

CALCULATION: 2 atoms of iodine correspond to 1 molecule of aldehyde and 2 molecules of formaldehyde to 3 molecules of lactic acid.

II. Friedemann Manganese Dioxide Modification. Friedemann, Cotonio, and Shaffer [56] (Washington University, St. Louis, and University of Chicago) employed successfully, for the oxidation of the lactic acid, phosphoric acid and manganese sulfate in conjunction with potassium permanganate and devised a special apparatus assembly. Friedemann and Kendall [57] substituted the highly active colloidal manganese dioxide for



Courtesy of the Authors and J. Biol. Chem. 1927, **73,** *335*

FIG. 168. Friedemann, Cotonio, and Shaffer Lactic Acid Apparatus.

the permanganate and Friedemann and Graeser [58] adopted some of the foregoing improvements and added others, including standardization with lactic acid from lithium lactate. They also embodied features of the Boas method.[59]

APPARATUS (Fig. 168). The *Friedemann, Cotonio, and Shaffer Apparatus* consists essentially of (1) a 300-ml. *Kjeldahl flask* fitted with a double-bored stopper carrying a *funnel tube* for introducing the oxidizing agent and a *Hopkins inner cooled reflux*

condenser connected by a side tube through a double-bored stopper with (2) a 150-ml. *receiving flask* in which the aldehyde is absorbed by bisulfite solution. Through the second hole of the stopper of the 150-ml. flask passes the narrowed end of a bead tower connected with a water pump for aeration by pressure.

REAGENTS. *Acid Zinc Sulfate Reagent.* Dissolve 14 g. of $ZnSO_4 \cdot 7H_2O$ in 1 liter of 0.1 N H_2SO_4.

Standard Sodium Hydroxide Solution. Adjust so that 20 ml. imparts a distinct alkaline color to phenolphthalein when added to 70 ml. of the zinc sulfate reagent.

Lactic Acid Standard, 0.1 M. Prepare lithium lactate ($LiC_3H_5O_3$) as follows. Mix 85% lactic acid with an equal volume of water, add a few drops of phenol red indicator, and saturated (about 20%) LiOH solution (in preference to Li_2CO_3 solution) in slight excess, as shown by the indicator. Heat to boiling, add alkali again to slight alkalinity, cool, add 4 volumes of ethanol, and cool further until the crystals separate. Collect the crystals on a Büchner funnel and wash well with ethanol. Recrystallize from water and dry at 100°.

Transfer 9.6 g. of $LiC_3H_5O_3$ to a 1-liter volumetric flask and add H_2SO_4 to 0.2 N final concentration when diluted to the mark. This 0.1 M standard solution will keep 1 year, if protected from the light or stored in a refrigerator.

Starch Indicator. Suspend 5 g. of arrowroot starch in 10 to 20 ml. of cold water and pour into 500 ml. of boiling water. Add 500 ml. of warm water and boil for 15 minutes. Cover the flask, cool, and keep in the refrigerator. Use the supernatant liquid only.

Oxidizing Agent. Colloidal MnO_2, 0.01 to 0.02 N.[60] $KMnO_4$ solution (3.1 g. per liter) is less satisfactory.

Phosphoric Acid-Manganese Sulfate Reagent. Dissolve 100 g. of $MnSO_4 \cdot 4H_2O$ in 500 ml. of warm water, add 25 ml. of 85% H_3PO_4, cool, and dilute to 1 liter.

Iodine Solution, strong. Dissolve 40 g. of iodine and 75 g. of KI in a small amount of water and dilute to 2 liters.

Standard Iodine Solution. Dilute a portion of the strong solution to 0.002 or 0.01 N or preferably, because of greater permanence, liberate iodine from 0.1 N iodate plus potassium iodide solution; 0.1 N $KH(IO_3)_2$ solution contains 3.2496 g. per liter and 0.1 N KIO_3 solution contains 3.567 g. per liter. Place 5 to 10 g. of KI, about 200 ml. of cold water, and 2 to 5 ml. of 5 N H_2SO_4 in a volumetric flask, add standard iodate, and dilute nearly to the mark with cold water. Warm to 20° and adjust to the mark. Keep in a cool place, remembering that iodine is volatile, even in dilute solution.

PROCESS. *Zinc Precipitation.* Add to 10 ml. of the milk exactly 70 ml. of *acid zinc sulfate reagent,* then run in exactly 20 ml. of *standard sodium hydroxide solution.* Mix well and centrifuge (preferably) or filter (Somogyi method).

Copper-Lime Precipitation. Transfer a 50-ml. aliquot of the clear solution to a 250-ml. volumetric flask, add 10 ml. of *20% copper sulfate solution* and 10 ml. of *20% calcium hydroxide suspension.* Mix, make up to volume, and centrifuge (Van Slyke method).

Oxidation. Place 10 ml. of *phosphoric acid-manganese sulfate reagent* and a pinch of *talcum powder* in a 300-ml. Kjeldahl flask, add 25 ml. of the clear solution (= 0.5 ml. of milk) from a pipet, make up to 100 ml., and attach to the apparatus. Place 10 ml. of *1.25% sodium bisulfite solution* in the 150-ml. extraction flask, and adjust the burner so as to boil in about 3 minutes. Add the *oxidizing agent* in portions through the funnel tube as soon as a vapor begins to pass into the condenser, continuing the addition so that it is always in excess as indicated by the brown or brown-red (not gray) color. Usually from 25 to 40 ml. of the oxidizing agent are re-

quired and 15 minutes are sufficient for complete oxidation. Toward the end of the oxidation, detach the receiving flask from the stopper and lower so as to collect the drip. Rinse with a small amount of water (total solution 50 to 75 ml.), remove the receiving flask, and cool in water to below 25°. The reaction of the acetaldehyde with the bisulfite is as follows:

$CH_3 \cdot CHO + S($

$$CH_3 \cdot CHOH \cdot SO_3Na$$

Titration. Add 1 to 2 ml. of clear *starch solution* and *strong iodine solution* in slight excess, then at once *thiosulfate solution* to combine with the iodine. Wash down the walls and adjust the end-point by *standard iodine solution.* Return the flask to the cooling bath, add 15 ml. of *saturated sodium bicarbonate solution* and titrate the bound bisulfite by adding *standard iodine solution* rapidly at a rate to keep pace with the decomposition of aldehyde and bisulfite. When the reaction slows up, add 1 ml. of *10% sodium carbonate solution* and continue the addition of *standard iodine solution* to the end-point as evidenced by the formation of a faint blue color persisting for 30 seconds.

Blank. Conduct a blank determination with pure water, using the filter paper and reagents. The blank result should be nearly proportional to the concentration of the iodine solution used.

CALCULATION. Each milliliter of 0.01 *N* iodine solution used in the titration of the bound sulfite is equivalent to 0.45 ml. of lactic acid.

NOTES. Meyer [61] describes a method employing the salient features of the von Fürth and Charnass method and the modification of Friedemann and associates.

Collazo and Ruiz [62] endorse the use of manganese sulfate as employed in the Friedemann modification, stating that it shortens the time for the oxidation and increases the yield.

III. Lieb and Zacherl Modification.[63] This modification differs from the Friedemann, Cotonio, and Shaffer modification chiefly in the form of the special aeration apparatus which is of Jena glass with ground-glass stoppers and fused-glass connections. Some of the special features of the apparatus are (1) the fused-in dropping funnel, (2) the capillary constriction of the air entrance tube designed to insure greater velocity of the current, thus preventing escape of acetaldehyde, (3) the condenser with outer and inner cooling surfaces, the narrow space (2 or 3 mm.) between, and the short cooling mantle (15 cm.), and (4) the glass frit plate that breaks the acetaldehyde into minute bubbles within the constricted inner portion (5 ml. capacity) of the receiver.

Hartmann and Hillig Oxalic Acid Volumetric Method.[64] The oxalic acid method is more time consuming than the aldehyde method, but has the advantage of simplicity and, in the experience of Hart, an associate of Hartmann and Hillig in the U. S. Food Administration, yields more accurate results.

APPARATUS. *Modified Palkin, Murray, and Watkins Extractor for Heavy Solvents.* The outer and inner tubes are respectively 2.5 and 2 cm. in diameter and the condenser is of the Liebig type. The original forms for heavy and light solvents are shown in Figs. 169 and 170. All-glass forms are also illustrated in the original paper.

REAGENTS. *Casein Precipitant.* Dissolve 4 g. of oxalic acid and 5 g. of potassium acetate in water, add 3 ml. of glacial acetic acid, and dilute to 200 ml. with water.

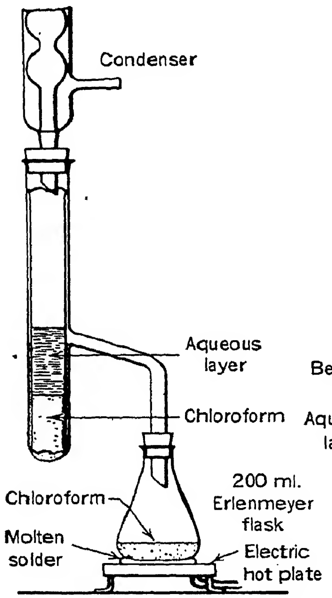
Lead Acetate Solution. Dissolve 75 g. of $Pb(CH_3COO)_2 \cdot 3H_2O$ in water, add 1 ml. of glacial acetic acid, and dilute to 250 ml. with water.

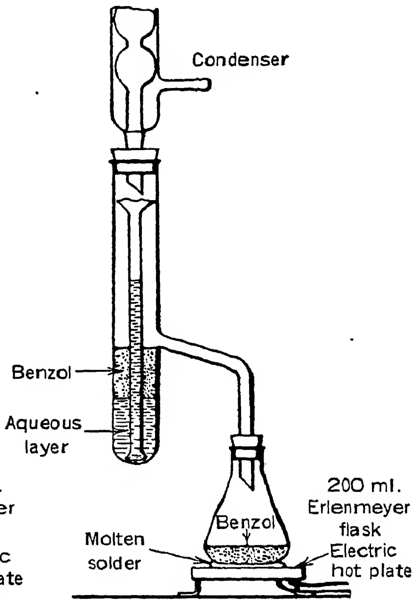
Ether, alcohol-free, washed three times with water.

Potassium Permanganate Solution, 5%. Standard solution prepared by dissolving 3.5424 g. of $KMnO_4$ in water and diluting to

1 liter; 1 ml. = 5 mg. of anhydrous oxalic acid.

Asbestos, purified. Digest asbestos with strong $KMnO_4$ in H_2SO_4 on a steam bath for several hours, pour on a Büchner funnel, and wash with water several times. Transfer to a large beaker, add dilute H_2SO_4 and sufficient $FeSO_4 \cdot 7H_2O$ solution to decompose the





Courtesy of Ind. Eng. Chem. 1925, 17, 612

FIG. 169. Palkin Extractor for Heavy Solvents.

FIG. 170. Palkin Extractor for Light Solvents.

Wash with hot water until 100 ml. of the filtrate require not more than 1 drop of standard 0.1 *N* $KMnO_4$ solution to produce a pink color.

PROCESS. *Clarification.* Place in a 250-ml. volumetric flask 50 g. of milk (or 6 g. of milk powder with 44 ml. of water), add 100 ml. of hot water (75°) and 10 ml. of *casein precipitant*, and heat on the steam bath for 15 minutes with occasional shaking. Cool, make up to the mark with water, shake, and filter. If the milk has not coagulated, add more *potassium acetate* and *acetic acid.*

Removal of Oxalic, Citric, and Phosphoric Acids and Albumin. Heat on the steam bath to about 50° 225 ml. of the above filtrate in a 250-ml. volumetric flask, add 5 ml. of *normal lead acetate solution,* shake, return to the steam bath for 30 minutes, cool, make up to the mark, and filter. Run in a rapid stream of *hydrogen sulfide gas*, stopper the flask, shake well, and filter.

Ether Extraction. Evaporate on a wire gauze 200 ml. of the clear filtrate in a 400-ml. beaker to about 5 ml. and transfer to the tube of the extractor, rinsing with smallest amount of water. Place the tube in a 1.5-liter beaker of boiling water and evaporate to a volume of about 10 ml. with aeration. Close the neck of the extractor tube with a

rubber stopper holding a $\frac{3}{16}$-inch glass tube long enough to reach nearly to the bottom of the liquid, and apply suction to the side arm of the extractor. Rinse the aspirator tube with about 5 ml. of water, add 1 ml. of *hydrochloric acid*, and mix. Insert the inner tube, connect with the condenser, and extract with *washed ether* for 3 hours, using a 20-inch condenser of the Liebig type. Place about 150 ml. of *washed ether* in a 300-ml. Erlenmeyer receiving flask, in which is suspended a strong thread to assure uniform boiling. Cover all corks with tinfoil and boil briskly on an electric hot plate.

Lactic Acid Oxidation. Add about 10 ml. of water to the extract and remove the ether by heating on a steam bath. Transfer the residue to a 600-ml. beaker with about 100 ml. of water, evaporate on a wire gauze to about 10 ml., add about 100 ml. of water, 10 ml. of *30% sodium hydroxide solution*, and 50 ml. of *strong potassium permanganate solution*, then heat to about 80°. Place the beaker in a briskly boiling water bath for 30 minutes. Cool, add with constant stirring 0.5 ml. less of *glacial acetic acid* than is required to neutralize the 10 ml. of sodium hydroxide added in the oxidation.

Manganese Precipitation. Place one-half of the mixture in a 250-ml. volumetric flask and run in *hydrogen sulfide* until a yellow color suddenly appears, then immediately add the remaining portion of the mixture to the flask, rinsing with a little water, shake vigorously, and allow to settle. If the turbid supernatant liquid is yellow, add more *strong potassium permanganate solution*, mix, and allow to settle, repeating until colorless. If pink in color, add a small amount of *hydrogen sulfide water* until the color is discharged. Cool, mix, make up to the mark, and filter.

Calcium Oxalate Precipitation. Place 200 ml. of the clear filtrate in a 400-ml. beaker and neutralize to *phenolphthalein* with *glacial acetic acid*. Add 5 drops of *acid* in excess. Heat to boiling, add 2.5 ml. of *calcium acetate solution*, boil for a few minutes, and allow to stand overnight. Filter on a thin asbestos pad in a Gooch crucible and wash with water.

The precipitation of the calcium oxalate may be expedited by shaking in an Erlenmeyer flask for 10 minutes with a handful of glass beads, cooling in a refrigerator for 30 minutes and again shaking for 10 minutes.

Permanganate Titration. Place the asbestos pad and precipitate in the original beaker, add about 150 ml. of water and 10 ml. of *1 + 1 sulfuric acid*, heat to about 80°, and titrate with *standard potassium permanganate solution*, of which 1 ml. is equivalent to 5 mg. of lactic acid.

CALCULATION. Obtain the grams of lactic acid (L) in the portion used for the analysis by the following formula:

$$L = \frac{0.005P}{0.576}$$

or

$$L = 0.00868P$$

in which P is the milliliters of permanganate solution used for the titration and 0.576 is the dilution factor ($\frac{1}{250}\times\frac{225}{250}\times\frac{200}{250}\times 200$).

Hillig Ferric Chloride Colorimetric Method.[65] APPARATUS. *Modified Palkin Extractor for Heavy Solvents.* See Hartmann and Hillig Method above.

Nessler Tubes, Photometer, or *Colorimeter.*

REAGENTS. *Ferric Chloride Reagent.* Dissolve 2 g. of $FeCl_3 \cdot 6H_2O$ in water, add 5 ml. of N HCl, and dilute to 200 ml.

Purified Carbon. Heat on a steam bath for 20 minutes with continual agitation a mixture of 10 g. of high grade carbon, 200 ml. of water, and 30 ml. of N HCl. Filter and collect the carbon on a Büchner funnel. Suck dry and tamp. Return the carbon to the beaker together with 200 ml. of water, mix, and filter. Repeat the treatment twice, then dry the purified carbon in a boiling water oven.

Standard Lithium Lactate Solution. Heat to boiling a mixture of 1 volume of sirupy lactic acid and 2 volumes of water, add

Li_2CO_3 in small portions until neutralized or slightly acid to phenol red. Evaporate on the water bath until the lithium lactate begins to crystallize, then add 5 volumes of ethanol and allow to stand overnight. Collect the crystals on a Büchner funnel and wash several times with ethanol. Recrystallize and dry in a boiling water oven.

Prepare the standard solution by dissolving 106.6 mg. of the lithium lactate in water and diluting to 100 ml.

PROCESS. *Clarification.* Weigh 50 g. of milk; disperse in 50 ml. of water 5 g. of dried milk product, 20 g. of cream or ice cream, or 25 g. of evaporated or condensed milk. Add to the liquid with constant stirring 6 ml. of *1.0 N sulfuric acid,* mix, then add *20% phosphotungstic acid solution* in the following amounts: milk, or dried milk, evaporated milk, or condensed milk dispersion, 5 ml.; cream, 1 ml.; ice cream, 2 ml. Transfer the mixture to a 100-ml. volumetric flask, rinsing with water and breaking up foam with a few drops of *ethanol* and a stirring rod. Make up to the mark, shake, allow to settle several minutes, and filter through a dry folded paper.

Ether Extraction. Pipet 50 ml. of the filtrate into the inner tube of a continuous extractor, add 0.5 ml. of *1 + 1 sulfuric acid,* and extract for 2 hours with 200 ml. of *washed ether* in which is suspended a piece of thread to secure uniform boiling. Adjust the heat so as to deliver a steady stream of ether without overheating. Regurgitation is avoided if the outlet of the condenser is not less than 0.5 inch (12 mm.) in diameter.

Dispersion in Water. Add to the flask containing the ether extract 20 ml. of water, heat cautiously on a steam bath to expel the ether, and neutralize to *phenolphthalein* with *saturated barium hydroxide solution.* Transfer to a 110-ml. volumetric flask, rinsing and diluting to about 90 ml. with *ethanol.* Heat on the steam bath nearly to boiling, cool, fill to the mark with *ethanol,* mix, and filter through a dry quantitative paper.

Pipet 100 ml. of the filtrate into a beaker and evaporate over a free flame to about 10 ml. Add 50 ml. of water and evaporate again to about 10 ml., then add from a buret 3.3 ml. of *0.1 N hydrochloric acid,* transfer to a 55-ml. volumetric flask, rinse with water, and dilute to about 40 ml.

Decolorization. Add to the solution 200 mg. of *purified carbon,* shake without delay, heat on the steam bath for 10 minutes with frequent mixing, cool, dilute to the mark, and filter through a dry quantitative paper, pouring back until clear.

Preliminary Color Comparison. Pipet 10 ml. of the clear decolorized filtrate into a 50-ml. Nessler tube, add 2.4 ml. of *0.1 N hydrochloric acid,* and dilute to about 40 ml. with water. Enclose in a jacket of black paper, add from a pipet 5 ml. of *ferric chloride solution,* fill to the mark, and mix. Pipet 5 ml. of the *standard lithium lactate solution* into another 50-ml. Nessler tube, add 3 ml. of *0.1 N acid* and 5 ml. of *ferric chloride reagent,* and fill to the mark.

Compare the color of the two solutions and calculate the approximate number of milligrams of lactic acid in each milliliter of the decolorized filtrate of the unknown.

Final Color Comparison. Pipet into a 50-ml. Nessler tube an even number of milliliters of the decolorized filtrate of the unknown containing less than 10 ml. of lactic acid. Also prepare a new *standard solution* from the stock solution by treatment of a suitable aliquot with *decolorized carbon,* diluting to 50 ml. and filtering as in the actual analysis, then pipet 10 ml. into a second Nessler tube. Treat both solutions as in the preliminary examination and compare the color.

CALCULATION. Obtain S, the volume in milliliters of the insoluble solids, by the following formula:

$$= \frac{50}{100}\left(\frac{F}{0.9} + \frac{P}{1.3}\right.$$

in which F is the per cent of fat and P the per cent of protein in the sample as given in the following compilation.[66]

	Fat %	Protein %
Whole milk	3.62	3.55
Dry whole milk	26.0	27.2
Evaporated whole milk	8.63	7.27
Skim milk	0.15	3.72
Dry skim milk	1.00	37.4
Sweetened condensed milk	9.28	8.36
Dry buttermilk	5.87	38.74

Calculate P, the per cent of lactic acid, from the final comparison by the following formula:

$$P = \frac{W}{100S} \times \frac{50}{110} \times \frac{100}{55} \times A$$

in which S is the milliliters of insoluble solids solution, W is the weight in grams of the sample, and A is the aliquot in milliliters taken for the final color comparison.

Note. Instead of the direct comparison with the standard solution, comparison of photometric readings in a 4-inch cell may be made with a curve prepared from the results of a series of readings on standard solutions of progressive dilutions.

Examples. Whole milk, wagon delivery, 0.0021 to 0.0056; evaporated milk, commercial, 0.0062 to 0.043; sweetened condensed milk, commercial, 0.0009 to 0.0012; dried whole milk, commercial, 0.0218 to 0.0396; dried skim milk, authentic, 0.0238 to 0.0734, commercial, 0.0446 to 0.997; malted milk, commercial, 0.177 to 0.186; cream, 20%, authentic, 0.0013 to 0.0015, 30%, 0.0007 to 0.001; dried buttermilk, commercial, 0.850 to 0.866; and ice cream, commercial vanilla, 0.0130 to 0.0135, commercial vanilla maple nut, 0.0150; liquid chocolate milk, wagon delivery, 0.009 g. per 100 g.

Citric Acid

The *Henkel-Wöhlk Method*, in reality an isolation method, serves for approximate quantitative purposes. In the *Scheibe Volumetric Method*, the reaction of citric acid with dichromate and of the latter with Mohr's salt are employed. The *Stahre pentabromacetone reaction* and the *Denigès acetone dicarboxylic acid reaction* both form the basis of qualitative and quantitative methods.

Henkel-Wöhlk Crystallization Gravimetric Method.[67] Process. *Coagulation.* Heat to 30° 10 liters of skim milk, from which the cream has been separated by the centrifugal process, add *rennet*, let stand some hours, and filter through linen. To the clear serum, add a few grams of *acetic acid*, boil for 30 minutes, and during the boiling add 50 to 70 g. of *fuller's earth* made into a thin paste with water.

Lead Precipitation. Filter off the coagulated portion and add to the filtrate *lead acetate* until a precipitate no longer forms, and again filter, then wash by decantation with cold water. Suspend the precipitate in water and decompose in a stream of *hydrogen sulfide gas*, passed through the suspension at room temperature for 3 hours with agitation. Filter and run a stream of air through the liquid to remove the hydrogen sulfide. Evaporate the filtrate on the water bath to a slimy brown sirup.

Ether Extraction. Mix the residue with *sand*, moisten with *absolute ethanol*, and extract with *ether* in a continuous extractor for 11 hours. Evaporate the ether, digest the aqueous liquid with *bone black*, and filter.

Crystallization. Evaporate to a sirup and separate the citric acid by repeated crystallizations, dry, and weigh.

Scheibe Dichromate Volumetric Method.[68] The somewhat involved and inconsistent instructions for this standard method, as given in the original paper and in König's treatise, appear in simplified form below. The basic reaction on which Scheibe depends is in accordance with the following equation:

$$C_6H_8O_7 + 3K_2Cr_2O_7 + 12H_2SO_4 = 3K_2SO_4 + 3Cr_2(SO_4)_3 + 16H_2O + 6CO_2$$

The excess of dichromate is reduced by Mohr's salt solution and the excess of this in turn is oxidized by titrating back with dichromate in accordance with the following equation, which, eliminating the inactive ammonium sulfate, is that involved in standardizing dichromate solution against ferrous sulfate:

$$K_2Cr_2O_7 + 6(NH_4)_2Fe(SO_4)_2 + 7H_2SO_4 \rightarrow Cr_2(SO_4)_3 + 3Fe_2(SO_4)_3 + 6(NH_4)_2SO_4 + K_2SO_4 + 7H_2O$$

Reagents. *Standard Potassium Dichromate Solution.* Dissolve 46.0 g. of crystallized $K_2Cr_2O_7$ in water and dilute to exactly 1 liter; 1 ml. = 10.01 mg. of anhydrous citric acid obtained by heating to constant weight at 40 to 50° citric acid crystals and cooling in a desiccator.

Standard Mohr's Salt Solution. Dissolve 150 g. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 700 ml. of water, add 100 ml. of H_2SO_4, and dilute to exactly 1 liter. Obtain the relative value of 20 ml. diluted with 80 ml. of water by titration against the dichromate solution of which theoretically 7.4 ml. are required for complete reaction.

Ethanolic Ammonia. Spirits of ammonia containing about 10% of gas and 80% of absolute ethanol.

Process. *Defecation.* Boil a mixture of 400 ml. of milk and 4 ml. of *2.5 N sulfuric acid,* add about 10 g. of *fuller's earth,* previously made into a thick paste with water, heat to boiling again, cool, filter, and wash into a 500-ml. volumetric flask, and fill to the mark. If the filtrate is not perfectly clear, mix with *dry fuller's earth* and filter through a dry paper.

Baryta Precipitation. To 100 ml. of the filtrate (= 80 ml. of milk), add *baryta water* equivalent to the sulfuric acid present (0.8 ml. of the 2.5 *N* solution), thus restoring the original acidity and avoiding decomposition of the citric acid by the mineral acid.

Concentration. Evaporate in a porcelain dish on a water bath to a sirup, stirring continually toward the end. Before the sirup is completely cold and the lactose solidifies, add 3.2 ml. of the *standard 2.5 N sulfuric acid* with stirring, thus insuring liberation of the citric acid.

Ethanol-Ether Extraction. Add gradually with stirring 20 ml. of *absolute ethanol* and, shortly after, 60 ml. of *ether.* Suck off the ethanol-ether liquid through an inverted small funnel, packed with cotton, attached by the stem to a filter pump, and wash the residue in the dish with *20 + 60 ethanol-ether* in the same manner. Transfer the clear liquid to a distillation flask, add *ethanolic ammonia* until a permanent turbidity appears, neutralize, and distil until the liquid is reduced to about 20 ml., thus avoiding the disturbing influence of the ether on the crystallization of the ammonium citrate.

First Ammonium Citrate Precipitation. Add to the residual liquid 60 ml. of *absolute ethanol,* heat on a water bath to boiling, and completely precipitate the ammonium citrate with 10 ml. of *ethanolic ammonia.* On standing several hours, the supernatant solution containing impurities becomes perfectly clear.

Second Ammonium Citrate Precipitation. Decant, add to the residue 1 ml. of *2.5 N sulfuric acid* and 1 ml. of water, then add 60 ml. of *absolute ethanol,* and reprecipitate with 10 ml. of *ethanolic ammonia.* After this second precipitation, the liquid, even on standing 24 hours, may not settle perfectly clear. In that case, addition of *ammonium carbonate* immediately after the ethanolic ammonia and refluxing is recommended. Filter on the same paper used after the first precipitation.

Oxidation. Dissolve the precipitate in 20 ml. of water and add from a buret 25 ml. of *standard dichromate solution,* then add 20 ml. of *sulfuric acid* and heat just short of boiling for 15 minutes. A reasonable excess of dichromate is essential for complete oxida-

tion. Use 30 ml. if the citric acid content of the milk exceeds 0.25%.

Titration. Cool, dilute with 50 ml. of water and add 40 ml. of *standard Mohr's salt solution.* If a blue precipitate does not form with *potassium ferricyanide indicator* (this may happen if the milk contains less than 0.10% of citric acid), add 10 ml. more of *Mohr's salt solution.* Titrate back with *standard dichromate solution* to the disappearance of the blue color.

CALCULATION. Obtain the percentage of citric acid (P) by the formula

$$P = \frac{100 \times 0.01001(M - D)}{\ } = 0.01251(M - D)$$

in which D is the total number of milliliters of standard dichromate solution added as such and M is the number of milliliters of dichromate solution equivalent to the Mohr's salt solution added.

CHECK. Prepare a solution of 0.685 g. of *anhydrous citric acid* in water and dilute to 100 ml. in a volumetric flask. Remove 20 ml. (= 0.137 g. of citric acid), add 25 ml. of *standard dichromate solution,* 20 ml. of *sulfuric acid,* and heat just short of boiling for 15 minutes. Cool, dilute with 50 ml. of water and add 40 ml. of *Mohr's salt solution,* then titrate back with *dichromate solution,* using *potassium ferricyanide solution* as indicator. Theoretically 13.7 ml. of the standard dichromate solution are required by the 0.137 g. of citric acid taken.

Methods Employing the Stahre Reaction. Stahre[69] demonstrated that when citric acid is oxidized with potassium permanganate in the presence of bromine water there is formed a white precipitate soluble in ether which he believed is a bromine substitution product of acetone, since acetone is formed from citric acid by oxidation with permanganate.

Wöhlk,[70] who studied the various methods, demonstrated that the products formed with the permanganate are as shown in the following equation:

$$\underset{\text{Citric acid}}{COOH\cdot CH_2\cdot C(OH)(COOH)\cdot CH_2\cdot COOH} + O \rightarrow \underset{\text{Acetone dicarboxylic acid}}{COOH\cdot CH{:}C(OH)\cdot CH_2\cdot COOH} + CO_2 + H_2O$$

He further showed that the bromination takes place as follows:

$$\underset{\text{Acetone dicarboxylic acid}}{COOH\cdot CH{:}C(OH)\cdot CH_2\cdot COOH} + 10Br \rightarrow \underset{\text{Pentabromacetone}}{\begin{matrix} CHBr_2\cdot CO\cdot CBr_3 \\ \text{or} \\ CBr_2{:}C(OH)\cdot CBr_3 \end{matrix}} + 2CO_2 + 5HBr$$

Stahre-Wöhlk Test. To 5 ml. of the solution, containing less than 5 mg. of citric acid, add 2 to 4 drops of *0.1 N potassium permanganate solution* and heat at 30°. If a brown color or a small precipitate of manganous hydroxide appears, add 1 to 2 drops of *4% ammonium oxalate solution* and about 1 ml. of *10% sulfuric acid* to clear the solution. On addition of 1 or 2 drops of *bromine water,* a distinct turbidity, due to pentabromacetone, appears.

Hartmann and Hillig Pentabromacetone Gravimetric Method.[71] This method, based on the Stahre reaction, is the same as that devised by the same authors for the determination of citric acid in fruit products (Part II, D2), except that proteins are precipitated with phosphotungstic acid at the outset. It is a Tentative A.O.A.C. Method.

REAGENT. *Lead Acetate Solution.* Dissolve 75 g. of $Pb(C_2H_3O_2)_2\cdot 3H_2O$ in water, add 1 ml. of glacial acetic acid, and make up to 250 ml.

PROCESS. *Phosphotungstic Precipitation.* Weigh into a 150-ml. beaker 50 g. of milk (or an amount of evaporated or dry milk containing approximately the same amount of solids suitably diluted), add 100 mg. of *tartaric acid* and 6 ml. of *1.0 N sulfuric acid,* and

heat on a steam bath for 15 minutes. Without removing from the bath, add 3 ml. of *20% phosphotungstic acid solution*, stir well, and continue the heating 5 minutes. Transfer to a 250-ml. volumetric flask, cool, rinse the beaker, and make up to the mark with *ethanol*. Filter through a dry paper and pipet 200 ml. (equivalent to 40 g. of the milk) of the filtrate into a centrifuge bottle.

Lead Precipitation. Add to the aliquot, 10 ml. of *lead acetate solution*, shake well for 2 minutes, and centrifuge at 1000 r.p.m. for 15 minutes. Test a portion of the supernatant liquid with *lead acetate solution*. If a precipitate forms, add more *lead acetate solution* and repeat the whirling; if the precipitate tends to decant with the liquid, increase the time and speed of centrifuging. Drain for several minutes.

Lead Removal. Add 150 ml. of water to the lead precipitate in the centrifuge bottle, shake well, saturate with *hydrogen sulfide gas*, transfer to a 250-ml. volumetric flask, add water to the mark, shake, and filter through a dry paper.

Pipet 200 ml. of the filtrate into a 250-ml. Erlenmeyer flask and proceed as described in Part II, D2, Hartmann Scheme, Normal Citric Acid, beginning with Process.

Calculation. Obtain the grams of anhydrous citric acid (G) in the portion weighed for analysis by the formula

$$G = \frac{0.424(a - b)}{0.64}$$

in which 0.424 is the factor for converting pentabromacetone into anhydrous citric acid, and $a - b$ and 0.64 are respectively the weight of pentabromacetone in, and the dilution of, the last of the three aliquots.

I. Lampitt and Rooke Modification.[72] The essential features of this modification were adopted by Deysher and Holm as given below. The original directions differ in the following details: (1) the mixture after the addition of the reagents is allowed to stand only 1 hour at room temperature (not 1.5 to 2 hours) before adding the extra permanganate; (2) only 10, 10, and 5 ml. of cold water are used for washing; (3) the precipitate is dissolved out of the crucible with industrial ethanol, followed by 20-, 10-, and 10-ml. portions of ether and the loss in weight is taken as pentabromacetone; and (4) in the calculation formula $0.005V$ (not $0.0035V$) is used, that is, the correction is 5, not 3.5 mg.

II. Deysher and Holm Modification.[73] The changes noted above were considered warranted in view of the recovery of citric acid (99.20 to 100.37%) added to decitrated milk, as compared with that by the original Lampitt and Rooke modification of 93.06 to 97.13%.

Process. *Defecation.* Heat 150 g. of milk to 50 to 60° in a 250-ml. volumetric flask, add 25 ml. of *2% potassium oxalate solution*, and shake, then add 20 ml. of *1 + 1 sulfuric acid*, shake again, and allow to cool. Add 10 ml. of *10% phosphotungstic acid solution*, make up to the mark, shake, and filter through a dry paper.

Pentabromacetone Precipitation. To 50 ml. of the deproteinized solution, add 10 ml. of *1 + 1 sulfuric acid* and 5 ml. of *37.5% potassium bromide solution*, followed by *5% potassium permanganate solution* (usually 25 ml.) added dropwise from a buret with constant stirring until a brown color persists. Allow to stand 1.5 to 2 hours at room temperature. If the brown precipitate disappears, add more *permanganate solution*, remove the excess of permanganate with *20% ferrous sulfate in 1% sulfuric acid solution*, then store in the refrigerator at 1 to 3° overnight.

Filtration and Weighing. Collect the precipitate in a sintered glass crucible (10G4), wash with two 20-ml. and one 10-ml. portions of water at not over 3°; this should not rise above 7° at the end of the washing. Dry in a vacuum desiccator at not over 20° and weigh.

CALCULATION. Obtain the weight of anhydrous citric acid (C) by the formula

in which W is the weight of the precipitate and V is the original volume of the filtrate from the reaction mixture, not including the washings. Introduce a correction of 0.005 per 100 ml. of reaction mixture.

Methods Employing the Denigès Reaction. Denigès[74] published his reaction four years after Stahre did his. The white crystalline mercury double salt that forms, according to Denigès, has the formula $SO_4 : Hg_2O_2 : Hg, (H_4C_5O_5) \cdot Hg \cdot Hg \cdot (H_4C_5O_5)$, with the molecular weight 1419.27, hence the conversion factor is 0.271. The permanganate, acting on the citric acid alone, yields acetone dicarboxylic acid, $COOH \cdot CH : C(OH) \cdot CH_2 \cdot COOH$, which may be regarded as the intermediate compound.

Denigès-Wöhlk Test.[75] To the solution containing the citric acid or citrate, add one-twentieth of the volume (at least 0.5 ml.) of the *Denigès reagent* (5 g. of *red mercuric oxide* in 20 ml. of *sulfuric acid* and 100 ml. of water), heat to boiling, then add 3 to 10 drops of *1.0 N permanganate solution*.

Beau Mercuric Sulfate Gravimetric and Volumetric Methods.[76] REAGENTS. *Mercuric Sulfate Solution.* Dissolve 50 g. of HgO in 500 ml. of water, add gradually with shaking 75 ml. of H_2SO_4, make up to 1 liter, and filter.

Standard Potassium Cyanate Solution. Dissolve 13 g. of KCN in 1 liter of water. Titrate 10 ml. against standard $AgNO_3$ solution.

A. GRAVIMETRIC PROCESS. Dilute 50 g. of the milk sample contained in a 200-ml. volumetric flask with 75 ml. of water and add 50 ml. of the *mercuric sulfate solution* slowly with shaking. Fill to the mark, filter on a dry paper, and refilter on the same paper until the filtrate is clear.

To 100 ml. of the filtrate (= 25 ml. of milk), add *1% potassium permanganate solution* (5 to 10 ml.) dropwise, boiling after each addition, until the yellow-white precipitate quickly settles beneath a colorless liquid. Collect the precipitate on a Gooch crucible, wash free of sulfuric acid, dry at 100°, and weigh.

CALCULATION. Multiply the weight of the precipitate by 0.271, thus obtaining the weight of anhydrous citric acid in 25 ml. of the milk.

B. VOLUMETRIC PROCESS. Dissolve on the filter, in *hydrochloric acid*, the moist precipitate formed with *potassium permanganate solution* obtained as described above, and wash the filter twice with 75 to 100 ml. of water. Add 20 ml. of *ammonia water*, 10 ml. of *potassium cyanate solution*, and 10 drops of *potassium iodide solution indicator*, then titrate with *standard 0.1 N silver nitrate solution* until a faint cloudiness appears.

CALCULATION. Subtract the milliliters of standard silver nitrate solution required for the titration of the unknown from that required for the cyanate alone. The difference is the milliliters corresponding to the weight of the mercury. From the weight of the mercury, calculate the weight of citric acid in accordance with the formula of the mercury compound given above.

THIOCYANIC ACID

Orella Chromic Acid-Silver Nitrate Volumetric Method.[77] Although thiocyanates do not appear to be present in human milk, Orella found 4 to 8 γ/ml. in cow's milk as determined by the following method.

PROCESS. Acidify the milk with *phosphoric acid* and distil the preformed hydrocyanic acid into *sodium hydroxide solution*.

Add *chromic acid mixture* to the residue in the flask, thus converting the thiocyanic acid into hydrogen cyanide. Proceed as above with the distillation into *sodium hydroxide solution* and titrate the hydrogen cyanide.

If the Liebig method[78] is used, run in *0.01 N silver nitrate solution* from a micro pipet until a permanent white turbidity is formed; 0.1 ml. of $AgNO_3$ = 0.54 mg. of HCN = 1.18 mg. of HSC.

Sucrose

One of the fraudulent practices based on scientific principles that now are more of historic than practical interest is the addition of sucrose to watered milk to simulate the specific gravity of pure milk and the refraction of its serum. For the detection of sucrose added as such, apply the Rothenfusser or the Baier and Neumann method as described under Cream below.

Molisch-Romani Test.[79] Reagent. *α-Naphthol Solution*, 20%. Dissolve 20 g. of α-naphthol in ethanol and make up to 100 ml. with the same solvent.

Process. Add to 1 drop of milk 2 drops of *20% α-naphthol solution* and 3 ml. of *hydrochloric acid* and boil rapidly for 3 to 4 seconds. Sucrose (up to 3%) produces a red coloration, but dextrose and lactose only a pale yellow.

Shake the solution with chloroform. The color due to sucrose passes into the solvent, coloring it yellow, but the color due to the other two sugars does not dissolve.

Lactose

The approved methods depend on copper reduction or polarization, the former being the more accurate. Proteins and certain mineral constituents are first precipitated, fat being carried down with the precipitate, a suitable reagent being used which for copper reduction methods usually is cupric sulfate plus sodium hydroxide, thus forming cupric hydroxide, which may be supplemented by sodium fluoride to precipitate calcium; for polariscopic methods mercuric nitrite or iodide with or without phosphotungstic acid is used. Basic lead acetate solution has been abandoned because of inaccuracies it introduces.

Determination of lactose is not one of the usual routine processes carried out in the inspection laboratory. It serves in extended investigations of yearly production of individual cows or in apportioning the constituents of modified milk.

Soxhlet Copper Reduction Gravimetric Method.[80] Reagents. *Fehling-Soxhlet Solutions*. (1) Dissolve 34.64 g. of $CuSO_4 \cdot 5H_2O$ in water and dilute to 500 ml. (2) Dissolve 173 g. of crystalline Rochelle salt ($KNaC_4H_4O_6 \cdot 4H_2O$) and 50 g. of NaOH in water and dilute to 500 ml.

Process. *Clarification*. Dilute 25 g., or an accurately measured volume corresponding to that weight, contained in a 500-ml. volumetric flask, with 400 ml. of water, add 10 ml. of *Soxhlet copper sulfate solution*, 8.8 ml. of *0.5 N sodium hydroxide solution*, make up to the mark, shake, allow the precipitate to settle, and filter through a dry paper. The solution should have an acid reaction and contain a small amount of copper in solution.

Copper Reduction. Mix a 100-ml. aliquot of the clarified and filtered milk with 25 ml. each of the two *Fehling-Soxhlet solutions*, heat to boiling, and continue the boiling exactly 6 minutes. Without delay, collect the cuprous oxide on a Gooch crucible, wash, dry, reduce to metallic copper by ignition in a stream of *hydrogen*, cool while continuing the hydrogen current, and weigh.

If preferred, the cuprous oxide may be weighed as such or after ignition as cupric oxide as described in Part I, C6*a*.

Calculation. Find the weight of lactose corresponding to the weight of copper in the Soxhlet-Wein table given below and calculate the percentage.

Scheibe Modification.[81] The only material difference from the original Soxhlet method is the addition of 20 ml. of a cold *saturated sodium fluoride solution* to the milk, supplementing copper sulfate and sodium hydroxide in the clearing. It is claimed that a minus error of about 0.10% is thus obviated.

LACTOSE FROM COPPER AND CUPROUS OXIDE (SOXHLET-WEIN)

Cu	Cu_2O	Lactose	Cu	Cu_2O	Lactose	Cu	Cu_2O	Lactose	Cu	Cu_2O	Lactose
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
101	113.7	72.4	146	164.4	105.8	191	215.0	140.0	236	265.7	173.9
102	114.8	73.1	147	165.5	106.6	192	216.2	140.8	237	266.8	174.6
103	116.0	73.8	148	166.6	107.3	193	217.3	141.6	238	268.0	175.4
104	117.1	74.6	149	167.7	108.1	194	218.4	142.3	239	269.1	176.2
105	118.2	75.3	150	168.9	108.8	195	219.5	143.1	240	270.2	176.9
106	119.3	76.1	151	170.0	109.6	196	220.7	143.9	241	271.3	177.7
107	120.5	76.8	152	171.1	110.3	197	221.8	144.6	242	272.5	178.5
108	121.6	77.6	153	172.3	111.1	198	222.9	145.4	243	273.6	179.3
109	122.7	78.3	154	173.4	111.9	199	224.0	146.2	244	274.7	180.1
110	123.8	79.0	155	174.5	112.6	200	225.2	146.9	245	275.8	180.8
111	125.0	79.8	156	175.6	113.4	201	226.3	147.7	246	277.0	181.6
112	126.1	80.5	157	176.8	114.1	202	227.4	148.5	247	278.1	182.4
113	127.2	81.3	158	177.9	114.9	203	228.5	149.2	248	279.2	183.2
114	128.3	82.0	159	179.0	115.6	204	229.7	150.0	249	280.3	184.0
115	129.6	82.7	160	180.1	116.4	205	230.8	150.7	250	281.5	184.8
116	130.6	83.5	161	181.3	117.1	206	231.9	151.5	251	282.6	185.5
117	131.7	84.2	162	182.4	117.9	207	233.0	152.2	252	283.7	186.3
118	132.8	85.0	163	183.5	118.6	208	234.2	153.0	253	284.8	187.1
119	134.0	85.7	164	184.6	119.4	209	235.3	153.7	254	286.0	187.9
120	135.1	86.4	165	185.8	120.2	210	236.4	154.5	255	287.1	188.7
121	136.2	87.2	166	186.9	120.9	211	237.6	155.2	256	288.2	189.4
122	137.4	87.9	167	188.0	121.7	212	238.7	156.0	257	289.3	190.2
123	138.5	88.7	168	189.1	122.4	213	239.8	156.7	258	290.5	191.0
124	139.6	89.4	169	190.3	123.2	214	240.9	157.5	259	291.6	191.8
125	140.7	90.1	170	191.4	123.9	215	242.1	158.2	260	292.7	192.5
126	141.9	90.9	171	192.5	124.7	216	243.2	159.0	261	293.8	193.3
127	143.0	91.6	172	193.6	125.5	217	244.3	159.7	262	295.0	194.1
128	144.1	92.4	173	194.8	126.2	218	245.4	160.4	263	296.1	194.9
129	145.2	93.1	174	195.9	127.0	219	246.6	161.2	264	297.2	195.7
130	146.4	93.8	175	197.0	127.8	220	247.7	161.9	265	298.3	196.4
131	147.5	94.6	176	198.1	128.5	221	248.7	162.7	266	299.5	197.2
132	148.6	95.3	177	199.3	129.3	222	249.9	163.4	267	300.6	198.0
133	149.7	96.1	178	200.4	130.1	223	251.0	164.2	268	301.7	198.8
134	150.9	96.9	179	201.5	130.8	224	252.4	164.9	269	302.8	199.5
135	152.0	97.6	180	202.6	131.6	225	253.3	165.7	270	304.0	200.3
136	153.1	98.3	181	203.8	132.4	226	254.4	166.4	271	305.1	201.1
137	154.2	99.1	182	204.9	133.1	227	255.6	167.2	272	306.2	201.9
138	155.4	99.8	183	206.0	133.9	228	256.7	167.9	273	307.3	202.7
139	156.5	100.5	184	207.1	134.7	229	257.8	168.6	274	308.5	203.5
140	157.6	101.3	185	208.3	135.4	230	258.9	169.4	275	309.6	204.3
141	158.7	102.0	186	209.4	136.2	231	260.1	170.1	276	310.7	205.1
142	159.9	102.8	187	210.5	137.0	232	261.2	170.9	277	311.9	205.9
143	161.0	103.5	188	211.7	137.7	233	262.3	171.6	278	313.0	206.7
144	162.1	104.3	189	212.8	138.5	234	263.4	172.4	279	314.1	207.5
145	163.2	105.1	190	213.9	139.3	235	264.6	173.1	280	315.2	208.3

Lactose from Copper and Cuprous Oxide (Soxhlet-Wein)—*Concluded*

Cu	Cu_2O	Lactose	Cu	Cu_2O	Lactose	Cu	Cu_2O	Lactose	Cu	Cu_2O	Lactose
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
281	316.4	209.1	311	350.1	232.9	341	383.9	256.5	371	417.7	281.4
282	317.5	209.9	312	351.3	233.7	342	385.0	257.4	372	418.8	282.2
283	318.6	210.7	313	352.4	234.5	343	386.2	258.2	373	420.0	283.1
284	319.7	211.5	314	353.3	235.3	344	387.3	259.0	374	421.1	283.9
285	320.9	212.3	315	354.6	236.1	345	388.4	259.8	375	422.2	284.8
286	322.0	213.1	316	355.8	236.8	346	389.6	260.6	376	423.3	285.7
287	323.1	213.9	317	356.9	237.6	347	390.7	261.4	377	424.5	286.5
288	324.2	214.7	318	358.0	238.4	348	391.8	262.3	378	425.6	287.4
289	325.4	215.5	319	359.1	239.2	349	392.9	263.1	379	426.7	288.2
290	326.5	216.3	320	360.3	240.0	350	394.0	263.9	380	427.8	289.1
291	327.4	217.1	321	361.4	240.7	351	395.2	264.7	381	429.0	289.9
292	328.7	217.9	322	362.5	241.5	352	396.3	265.5	382	430.1	290.8
293	329.9	218.7	323	363.7	242.3	353	397.4	266.3	383	431.2	291.7
294	331.0	219.5	324	364.8	243.1	354	398.6	267.2	384	432.3	292.5
295	332.1	220.3	325	365.9	243.9	355	399.7	268.0	385	433.5	293.4
296	333.3	221.1	326	367.0	244.6	356	400.8	268.8	386	434.6	294.2
297	334.4	221.9	327	368.2	245.4	357	401.9	269.6	387	435.7	295.1
298	335.5	222.7	328	369.3	246.2	358	403.1	270.4	388	436.8	296.0
299	336.6	223.5	329	370.4	247.0	359	404.2	271.2	389	438.0	296.8
300	337.8	224.4	330	371.5	247.7	360	405.3	272.1	390	439.1	297.7
301	338.9	225.2	331	372.7	248.5	361	406.4	272.9	391	440.2	298.5
302	340.0	225.9	332	373.8	249.2	362	407.6	273.7	392	441.3	299.4
303	341.1	226.7	333	374.9	250.0	363	408.7	274.5	393	442.4	300.3
304	342.3	227.5	334	276.0	250.8	364	409.8	275.3	394	443.6	301.1
305	343.4	228.3	335	377.2	251.6	265	410.9	276.2	395	444.7	302.0
306	344.5	229.1	336	378.3	252.5	366	412.1	277.1	396	445.9	302.8
307	345.6	229.8	337	379.4	253.3	367	413.2	277.9	397	447.0	303.7
308	346.8	230.6	338	380.5	254.1	368	414.3	278.8	398	448.1	304.6
309	347.9	231.4	339	381.7	254.9	369	415.4	279.6	399	449.2	305.4
310	349.0	232.2	340	382.8	255.7	370	416.6	280.5	400	450.3	306.3

Munson and Walker Method. Clarify as described above for the Soxhlet method, then proceed as directed in Part I, C6*a*.

Shaffer and Hartmann Copper Reduction Iodometric Method.[32] In connection with studies of the reversible reaction at Washington University Medical School, St. Louis, methods based on the following equation for both cuprous and cupric titration were devised:

Reagents. *Fehling-Soxhlet Solutions.* See Soxhlet Method above.

Iodate-Iodide Solution. Dissolve 5.4 g. of KIO_3 and 60 g. of KI in water, to which is added a small amount of alkali (to prevent the formation of hydriodic acid and its oxidation by air), then dilute to 1 liter.

Process. *Tungstate Clarification.* Pipet 10 ml. of milk and 80 ml. of water into a 200-ml. flask, then add from a pipet 5 ml. of *10% sodium tungstate solution* and 5 ml. of 0.213 *N sulfuric acid.* Shake and filter on a pleated paper; return to the paper if not clear.

Copper Reduction. Measure 25 ml. each of the two *Fehling-Soxhlet solutions* into a 300-

or 400-ml. flask, then pipet into the mixture 25 or 50 ml. of the milk filtrate, representing 2.5 or 5 ml. of milk, together with 25 ml. of water if only 25 ml. of the filtrate are added. Heat to boiling in 4 minutes and continue to boil 2 minutes, as directed for the Munson and Walker method, then cool in water for 3 to 4 minutes.

Cuprous Titration. Without filtering add to the cuprous precipitate from a pipet 50 ml. (or 25 ml. if the amount of cuprous oxide is small) of *iodate-iodide solution,* followed quickly by 15 to 17 ml. of *5 N sulfuric acid* delivered from a cylinder. The hypoiodite in alkaline solution oxidizes the organic acids and some iodine may be thus used if the acid is not added quickly. Shake gently for a few moments until the cuprous oxide has dissolved. The solution should become clear; often, however, some cuprous iodide separates. Add 20 ml. of *saturated potassium oxalate solution* and rotate the flask until any remaining cuprous oxide has completely dissolved.

If the alkaline solution is taken from the tap while it is still warm (above 40°) and the iodate-iodide, acid, and oxalate are added at once, the cuprous oxide dissolves almost immediately and the solution remains clear; when cold, cuprous iodide may first separate and when this is dissolved after addition of the oxalate, acid potassium tartrate may crystallize out, but neither separation affects the results.

Thiosulfate Titration. Titrate the solution with *0.1 N standard potassium thiosulfate solution,* adding as indicator *starch solution* before the disappearance of the green color.

Blank Titration. Conduct a blank determination, using 50 ml. of water in place of the lactose solution. The blank remains practically unchanged for a long period, provided the two Fehling-Soxhlet solutions are measured separately and mixed as used.

CALCULATION. Subtract from the number of milliliters used in the blank titration that used in the titration of the lactose solution. The remainder represents the I_2 required for the oxidation of the cuprous salt. Multiply this remainder by 6.36 which is the copper factor for 1 ml. of 0.1 *N* thiosulfate solution, then find the corresponding weight of lactose in the Munson and Walker table (Part I,

I. Blanchetière Modification.[83] In the modification designed primarily for dextrose, Blanchetière substituted *oxalic acid* for sulfuric acid and claimed that thereby liberation of iodine from the potassium iodide of the standard iodine solution is avoided.

II. Kometiani Modification.[84] The Blanchetière modification, with minor changes, is described by Kometiani of the Zootechnological Institute of Tiflis. Used in connection with the Blanchetière-Kometiani table, the procedure has at least the merit of simplicity.

REAGENTS. *Bertrand Copper Sulfate and Alkaline Rochelle Salt Reagents,* for copper reduction, Part I, C6*a*.

Standard Iodine Solution, 0.1 *N*. Use the equation: 1 ml. = 6.36 mg. of Cu.

PROCESS. *Clarification.* Pipet 10 ml. of milk into a 200-ml. volumetric flask and fill about one-third full with water, add 2 ml. of *1.0 N sodium hydroxide solution,* and titrate with *Bertrand copper sulfate solution* in the presence of 2 drops of *phenolphthalein solution* until the color changes from violet to light blue, thus precipitating protein and fat. Fill to the mark, shake, allow to stand until the liquid above is clear, then filter on a dry paper.

Copper Reduction. Place a 20-ml. aliquot of the clear filtrate in a 200-ml. Erlenmeyer flask, add 20 ml. of each *Bertrand solution,* also a bit of *tallow,* shake, heat to boiling, and boil for exactly 6 minutes.

Titration. Cool the solution, acidify with 40 ml. of *saturated oxalic acid solution,* add 20 to 25 ml. of *0.1 N iodine solution,* agitate, and allow to stand 2 to 3 minutes. Titrate back with *0.1 N thiosulfate* solution, using as indi-

cator *starch solution* added near the end of the titration. The added iodine solution must be about double that required for the reaction, that is, the thiosulfate solution required must be about half the volume of the iodine solution. The oxalic acid changes the color of the solution from dark to light blue, which with iodine solution changes to dark green.

Blank. Conduct a blank titration, using the same volume of iodine solution.

CALCULATION. Subtract from the number of milliliters used in the blank that used in the actual determination. The difference represents the lactose in terms of milliliter of thiosulfate solution. Find in the Blanchetière-Kometiani table herewith the number of milligrams of lactose corresponding to the number of milliliters of thiosulfate solution obtained by difference. Divide the number of milligrams of lactose by 10. The quotient is the grams of lactose in 100 ml. of milk.

adapted for the determination of lactose in milk by Gohr [87] who also contributed a calculation table. The basic reaction with standard ferricyanide in alkaline solution is

$$. + K_3Fe(CN)_6 \longrightarrow K_4Fe(CN)_6 + \cdots$$

The excess of ferricyanide reacts with potassium iodide in accordance with the Lenssen and Mohr equation:

$$2H_3Fe(CN)_6 + 3HI \rightleftharpoons 2H_4Fe(CN)_6 + I_2$$

Since the reaction is reversible, zinc sulfate is added, thus forming zinc ferrocyanide, then the liberated iodine is titrated with standard thiosulphate solution.

I. Gohr Modification.[87] Gohr (Bönn University) removes proteins and fat by ferrocyanide plus zinc sulfate in alkaline solution. Other details are essentially those of the original method.

LACTOSE FROM 0.1 N THIOSULFATE

Lactose	Thiosulfate	Lactose	Thiosulfate	Lactose	Thiosulfate	Lactose	Thiosulfate	Lactose	Thiosulfate	Lactose	Thiosulfate
mg.	ml.	mg.	ml.	mg.	ml.	mg.	ml.	mg.	ml.	mg.	ml.
11	2.48	26	5.75	41	8.91	56	11.98	71	15.00	86	17.94
12	2.71	27	5.97	42	9.11	57	12.19	72	15.19	87	18.14
13	2.92	28	6.19	43	9.32	58	12.39	73	15.39	88	18.33
14	3.14	29	6.40	44	9.53	59	12.59	74	15.58	89	18.54
15	3.36	30	6.62	45	9.73	60	12.80	75	15.79	90	18.73
16	3.58	31	6.82	46	9.95	61	13.10	76	16.00	91	18.91
17	3.81	32	7.04	47	10.16	62	13.19	77	16.18	92	19.12
18	4.03	33	7.24	48	10.36	63	13.39	78	16.37	93	19.31
19	4.25	34	7.44	49	10.56	64	13.60	79	16.57	94	19.50
20	4.47	35	7.64	50	10.77	65	13.78	80	16.78	95	19.69
21	4.69	36	7.86	51	10.97	66	13.99	81	16.97	96	19.89
22	4.89	37	8.08	52	11.18	67	14.19	82	17.17	97	20.08
23	5.10	38	8.29	53	11.38	68	14.40	83	17.36	98	20.27
24	5.33	39	8.51	54	11.59	69	14.59	84	17.56	99	20.47
25	5.53	40	8.71	55	11.78	70	14.80	85	17.75	100	20.66

Hagedorn and Jensen Ferricyanide-Iodide Volumetric Method.[85] Designed originally for the determination of reducing sugar in blood, the manipulation has been somewhat simplified by Issekutz and von Both [86] and

REAGENTS. *Alkaline Potassium Ferricyanide Solution,* 0.05 N. Dissolve 16.46 g. of $K_3Fe(CN)_6$ and 70 g. of anhydrous Na_2CO_3 in water and dilute to exactly 1 liter.

Iodide-Zinc Sulfate Reagent. Dissolve 25

g. of KI, 50 g. of $ZnSO_4 \cdot 7H_2O$, and 250 g. of NaCl in water and dilute to 1 liter.

PROCESS. *Clarification.* To 5 g., or a corresponding volume, of milk, contained in a 100-ml. volumetric flask, add 1 ml. each of *15% potassium ferrocyanide solution* and 30% *zinc sulfate solution,* make slightly alkaline to *phenolphthalein solution* with *sodium hydroxide solution,* dilute to the mark, shake, allow to settle 15 minutes, and filter through a dry paper.

Ferricyanide Reduction. Pipet 2 to 6 ml. of the clear filtrate into a 175-ml. Erlenmeyer flask, dilute to about 20 ml., then add 10 ml. of *0.05 N alkaline ferricyanide solution,* and heat on a boiling water bath for 20 minutes.

Thiosulfate Titration. Cool the solution, add 10 ml. of *iodide-zinc sulfate reagent* and 10 ml. of *9% acetic acid solution,* then titrate the liberated iodine with *standard 0.05 thiosulfate solution,* using *0.1% starch solution* as indicator.

Blank. Conduct a blank determination in the same manner as the actual analysis and make the necessary deduction.

CALCULATION. Find in the Gohr table the milligrams of lactose corresponding to the number of milliliters of standard ferricyanide consumed in the titration and from this calculate the weight of lactose in the portion used for analysis.

LACTOSE FROM POTASSIUM FERRICYANIDE (GOHR)

0.05 N $K_3Fe(CN)_6$	Lactose									
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
ml.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0	....	....	....	....	....	....	1.00	1.19	1.37	1.56
1	1.74	1.93	2.11	2.30	2.48	2.66	2.85	3.04	3.23	3.42
2	3.62	3.81	4.00	4.19	4.37	4.55	4.74	4.93	5.12	5.31
3	5.50	5.69	5.88	6.08	6.27	6.46	6.65	6.85	7.04	7.23
4	7.42	7.62	7.81	8.00	8.19	8.37	8.56	8.74	8.93	9.12
5	9.31	9.50	9.69	9.88	10.08	10.27	10.46	10.65	10.85	11.04
6	11.24	11.44	11.64	11.84	12.04	12.26	12.47	12.68	12.89	13.10
7	13.30	13.50	13.70	13.90	14.11	14.32	14.53	14.74	14.96	15.18
8	15.41	15.64	15.86	16.09	16.33	16.57	16.81	17.05	17.29	17.52
9	17.76	18.00	18.25	18.50	18.75	19.00	19.25	19.50	19.75	20.00

II. Blackwood Modification.[88] REAGENTS. *Ferricyanide Reagent.* Dissolve 8.25 g. of $K_3Fe(CN)_6$ and 10.6 g. of anhydrous $NaCO_3$ in water and dilute to 1 liter.

Iodide-Zinc Sulfate Reagent. See Gohr Modification above.

PROCESS. *Clarification.* Follow the Folin-Wu tungstic acid or the Fujita and Iwatake cadmiun sulfate method.

Ferricyanide Reduction. To 5 ml. of the *ferricyanide reagent* in a Pyrex test tube, add 5 ml. of the clarified solution of the sample containing 0.20 to 5 mg. of lactose and heat in a boiling water bath for 15 minutes. Cool for 3 minutes in running water and add 5 ml. of the *iodide-zinc sulfate reagent* and 3 ml. of *5% acetic acid.*

Titration. Using *1% starch solution* as indicator, added toward the end of the opera-

tion, titrate the liberated iodine with *standard 0.01 N thiosulfate solution.*

Wiley Polarimetric Method.[89] APPARATUS. *Polariscope.*

REAGENTS. *Acid Mercuric Nitrate Solution.* Dissolve metallic mercury in double its weight of HNO_3. Dilute the solution with an equal volume of water.

Mercuric Iodide Solution. Dissolve 33.2 g. of KI and 13.5 g. of $HgCl_2$ in a mixture of 20 ml. of strongest acetic acid and 640 ml. of water.

PROCESS. Weigh on a tared sugar tray or beaker 65.95 g. of the sample and transfer to a 100-ml. volumetric flask, rinsing with a little water. If preferred, measure into the flask from a buret 64 ml. (the volume of 65.95 g. of average milk) or a volume based on an actual determination of specific gravity in accordance with the following Quevenne readings: 24, 64.4 ml.; 26, 64.3 ml.; 28, 64.15 ml.; 30, 64.0 ml.; 32, 63.9 ml.; 34, 63.8 ml.; 36, 63.6 ml.

Clarification. Add to the weighed or measured portion of the sample as clarifier 1 ml. of *acid mercuric nitrate solution* or 30 ml. of *mercuric iodide solution* and shake. The use of 10 ml. of lead subacetate, first proposed as an alternate clarifier, introduces serious errors. Fill to the mark with water and add 2.6 ml. additional to compensate for the bulk of the precipitate, shake again, allow the precipitate to settle for a few minutes, and filter through a dry paper.

Polarization. Fill a 200-mm. tube and polarize in an instrument with the Soleil-Ventzke scale.

CALCULATION. The reading divided by 2 is the percentage of lactose.

A.O.A.C. Modification. The Wiley method, essentially as given above, was official for many years, but in the 1935 revision of the *Methods* the following changes were made in the interest of accuracy: (1) the volumes of milk specified (64.25 to 63.50) are in each case 0.15 ml. less than those given in the original method, (2) in preparing the acid mercuric nitrate solution the solution of metallic mercury in nitric acid is diluted with a five-fold volume of water and 18 to 20 ml. are used in the analysis, and (3) 5% phosphotungstic acid solution instead of water is used to dilute the liquid to 102.6 ml.

FERMENTATION ODORS

Milk often develops a disagreeable odor or flavor due to improper food or water or to careless handling. Ferments which cause these qualities, as well as those which result in an abnormal mechanical condition of the curd, are especially undesirable in cheese making. They are detected by the following tests.

Wisconsin Curd Test.[90] Fill sterilized pint fruit jars two-thirds full with the milk, cover, and heat in a water tank until the milk reaches 37° C. (98° F.). Add 10 drops of *rennet extract* and rotate until mixed. Allow to stand about 20 minutes until the curd is firm, then cut into small pieces with a sterilized case-knife and stir from time to time for 30 to 45 minutes. When the pieces of curd are firm, pour off the whey and digest as before for 6 to 12 hours, pouring off at intervals any remaining whey that may separate from the matted curd. Finally drain, cut the curd, and examine for a bad odor, or a spongy or otherwise abnormal texture due to gas formation.

The thermometer and knife should be carefully washed with hot water before being introduced into each sample.

Gerber and Walther Test. Fill sterilized 2.5-ml. test tubes three-fourths full with the milk, close with a tin cap or plug of sterilized cotton, digest in a water tank kept at 45°, and examine the contents after the sixth and twelfth hour.

Good milk should not develop a bad odor, bad taste, or bubbles of gas in 12 hours.

Enzymes

Babcock and Russell [91] describe a proteolytic enzyme (galactase) which aids in the ripening of cheese. A peptidase, an amylase (diastase), a lipase or fat-splitting enzyme, and an alcoholase are also present.

Numerous investigations have been devoted to enzymes, or groups of enzymes, the activities of which serve to distinguish raw from heated milk (peroxidase or oxidase, aldehyde reductase, phosphatase), bacterial pollution (reductase), or pus from diseased udders (catalase).

Peroxidase

Guaiac solution or various easily oxidizable substances in conjunction with hydrogen peroxide may be used in testing for peroxidase, an enzyme believed to be formed in the mammary glands. Milk heated below 78° reacts the same as raw milk with all the tests; milk heated at 80° or above reacts faintly or not at all. Addition of a small percentage of raw milk to milk heated above 80° may be readily detected. Kühn [92] finds that the guaiac test and La Wall [93] that the benzidine test may give the same reactions with milk preserved by hydrogen peroxide as with milk heated above 80°. Other preservatives interfere more or less with the tests according to the kind and amount.

Formerly, when pasteurizing at 78 to 80° was required, peroxidase tests were of forensic value; since milk heated below 78° reacts like raw milk. Now that pasteurization at 63° is deemed effective, Lythgoe [94] has shown that they are of no value for the purpose.

Violle [95] points out that raw milk may contain only traces of peroxidase and that milk heated at 78 to 80° may contain it in large amount owing to the fraudulent addition of vegetable materials such as potatoes, soy beans, and peas. As peroxidase is formed in the mammary glands, it may be present in abnormally large amount if the glands are diseased.

Arnold Guaiac Test.[96] To 10 ml. of milk in a test tube, add a few drops of *5 to 10% guaiac solution in ethanol or acetone*. Raw milk or milk heated below 80° gives a deep blue color in the course of a few minutes.

Tincture of guaiac is said to become active only after exposure to light and air,[97] whereas the acetone solution is active at once.[98]

Modified Arnold Guaiac Test. Mix 10 ml. of the milk with 1 or 2 drops of *3% hydrogen peroxide solution*, then allow 2 ml. of *5 to 10% guaiac solution* to run down the sides of the tube so that it forms a layer on top of the milk. A blue color develops if the milk is raw or not heated above 80°.

Dupouy Paraphenylenediamine Test. In 1897 Dupouy [99] showed that guaicol, paraphenylenediamine, hydroquinone, pyrocatechin, or α-naphthol, in conjunction with hydrogen peroxide, give peroxidase reactions with raw milk and may be used in tests for heated milk. This is also known as the Storch test, since that author [100] employed it in examining pasteurized milk and milk products which the Danish law required to be heated to at least 85° as a safeguard against tuberculosis. Lythgoe [101] finds that the test is worthless for distinguishing milk pasteurized at 63°, the temperature commonly employed in the holding process, from raw milk.

Process. To 5 ml. of the milk contained in a test tube, add 1 drop of *0.2% hydrogen peroxide solution* (containing 1 ml. of concentrated sulfuric acid per liter) and 2 drops of *2% paraphenylenediamine*. A blue coloration formed immediately indicates that the milk has not been heated to 78°; a blue-gray coloration formed immediately or within half a minute indicates probable heating to 79 to 80°; no change in color or a faint violet-red is evidence of heating above 80°.

The test may also be applied to cream, whey, buttermilk neutralized with lime wa-

ter, and the watery liquid obtained by melting butter, pouring off the fat, and diluting with an equal volume of water.

Rothenfusser Guaiacol-Paraphenylenediamine Test.[102] Prepare a serum by mixing 100 ml. of the milk with 6 ml. of *lead subacetate* and filtering at 30 to 40°.

To 10 ml. of the serum, add 1 to 2 drops of *0.3% hydrogen peroxide solution* and a few drops of a mixture of a solution of 1 g. of *paraphenylenediamine hydrochloride* in 15 ml. of water with a solution of 2 g. of *guaiacol* in 135 ml. of *ethanol*. With raw milk or heated milk containing a very small percentage of raw milk, a violet color develops.

It is claimed that the test is more delicate than those applied directly to the milk and that ordinary preservatives do not interfere.

Roi and Köhler Iodide-Starch Test.[103] The reaction on which this method is based was first employed by Storch but was abandoned as not satisfactory.

Shake 100 ml. of the milk with 1 ml. of *1% hydrogen peroxide solution*. To 3 ml. of the mixture, add 3 ml. of an *iodide-starch solution* (2 to 3 g. of potassium iodide dissolved in a few milliliters of water and mixed with 100 ml. of 2% starch paste) and shake vigorously. Raw milk becomes blue; milk pasteurized at high temperatures or boiled milk remains colorless.

Wilkinson and Peters Benzidine Test.[104] Add to 10 ml. of the milk 2 ml. of a *4% ethanolic solution of benzidine* and 2 to 3 drops of *glacial acetic acid*, just sufficient to coagulate the milk. Shake and add 2 ml. of *3% hydrogen peroxide solution*, allowing the reagent to run down the sides of the test tube. Raw milk or milk heated below 78° takes on an intense blue color, but milk heated at 80° or higher remains colorless.

NOTE. Leffmann[105] finds that certain photographic developers, such as *metaquinone* (Lumière) and *amidol* (Hauff), may be substituted for the reagents noted in the preceding tests.

REDUCTASE

Schardinger Methylene Blue Test.[106] The reaction is attributed to the action of bacteria and the test accordingly is regarded as a measure of bacterial contamination. Several well-known authors utilize the test as a measure of keeping quality. Viale[107] considers that the reaction is due to cysteine.

To 20 ml. of the milk, add 1 ml. of *M reagent* (a solution prepared by mixing 5 ml. of a standard ethanolic solution of methylene blue and 195 ml. of water). Heat on a water bath at 45 to 50° and note the time required for decolorization, which for sanitary milk should not be less than 1 hour.

Barthel[108] substitutes for ordinary methylene blue, which is the chloride, the *zinc double chloride*, but in other essential details adheres to the original Schardinger method. He states that milk which decolorizes the liquid in 1 hour is bacterially unfit for food, especially for infants, whereas that which reacts within 3 hours is of inferior quality.

Others[109] have proposed various modifications, differing chiefly in the method of preparing the reagent, the proportion of reagent to milk, and the temperature of the test. In order to prevent oxidation, Müller uses sufficient *olive oil* to form a layer 1 cm. thick on the surface. Barthel employs a *mineral oil*. The same result is attained by making the test in a Lobeck-Gerber tube which bears marks for measuring the reagent and milk and is provided at the narrowed lower end with a screw vent through which, on inverting, air is expelled by means of a rubber stopper, with a glass prolong, fitted to the other end.

Hastings and Davenport[110] consider that the test determines the keeping quality better than the bromothymol blue test[111] and the bromocresol purple test,[112] since it measures the bacterial content. Strohecker and Schnerb[113] give the following as the decolorization time at 60°: raw milk 1 to 2.75, milk

pasteurized at 63 to 65° 3 to 30, and at 59 to 61° 1.75 to 4 minutes. Powell, Jenkins, and Thomas [114] found that the reducing time was 3.5 hours longer for samples held overnight at 40° than for those held at 60° F. Sandberg [115] considers that the test estimates the bacterial content quite as well as plate count or microscopic examination.

On the other hand, Hanke [116] casts doubt on the value of the test because it does not take into account the types of bacteria present; furthermore, Fay and Aikins,[117] also Phillips, Ashton, Simons, and Thomas,[118] note that various factors, some (such as light) controllable, others (such as composition) not, influence materially the time of reduction. Results by Malcolm and Leitch [119] indicate that the test is not trustworthy under all conditions.

Wilson Modification.[120] Results by Williams, Davies, and Thomas [121] indicate that the time for decolorization, within 5.5 hours, of the Wilson modification is about the same as that of the original method.

Aldehyde Reductase

Schardinger Formaldehyde-Methylene Blue Test.[122] This test is chiefly useful as a means of distinguishing raw milk from that pasteurized at 63°.

Reagent. *FM*. This is the same as the *M* for the reductase test, except that 5 ml. of 40% formaldehyde are added and correspondingly less (190 ml.) of water is used.

Process. This also is the same, although several authors prefer to digest at 65 or 70° instead of at 45 to 50°.

Schardinger found that raw milk, tested soon after milking, decolorized the reagent in about 10 minutes. In Lythgoe's experiments,[123] raw milk decolorized the reagent in 5 minutes, whereas milk pasteurized commercially at 63° for 35 minutes and not over 2 days old did not react in several hours.

Frost Test.[124] This test, although not based on enzyme action, is here given as worthy of mention as a check on the aldehyde reductase method.

Mix a few milliliters of the milk with one-fifth as much of a *saturated methylene blue solution*. Allow to stand for 30 minutes, then centrifuge and spread the sediment on a slide. When dry, examine microscopically. The field in the case of raw milk is stained a uniform blue in which are fat globules or leucocytes forming clear areas. The polymorphonuclear cells are irregular in outline, 12 μ in diameter, and absorb little or no stain. In the sediment from milk heated to 60°, the polymorphonuclear leucocytes are rounded and shrunken to 8 μ with deeply stained nuclei.

Catalase

Catalase, also known as superoxidase, differs from oxidase in that it splits 2 molecules of hydrogen peroxide into 2 of water and 1 of oxygen. Roeser and Wassermann [125] showed that the gas is not pure oxygen and titration gives more accurate results than measurement of the gas.

The catalase reaction bears a more or less definite relation to the number of white blood corpuscles (leucocytes). These are present in all milk, but are more abundant in colostrum and milk from cows advanced in lactation and are very numerous in milk from diseased udders. Tests made soon after milking are useful in detecting milk of this character.

Koning Peroxide Volumetric Method.[126] Mix 15 ml. of the sample and 5 ml. of *1% hydrogen peroxide*. After allowing to stand for 2 hours at 22 to 30°, add 10 ml. of *hydrochloric acid* and 10 ml. of *10% potassium iodide solution* and allow to stand 15 minutes. Dilute with 10 ml. of water and titrate with *0.1 N thiosulfate solution*, using *starch paste indicator*.

Calculate the *catalase number*, which is the milliliters of hydrogen peroxide corresponding to 100 ml. of milk. The number for good milk should not exceed 110.

Koning Gasometric Method.[126] The process is the same as for the volumetric method, except that the reaction takes place in a Henkel tester and the volume of gas is measured. The limit for good milk is 4 to 5 ml.

Laxa Gasometric Method.[127] The apparatus is a Mohr pipet with a stopcock at the bottom of the suction tube.

Fill the pipet by suction with a mixture of 3 parts of milk and 1 part of *10% hydrogen peroxide solution*, close the stopcock, and suspend the tube over a beaker to catch such milk as is displaced by the gas as generated. The results agree closely with those by Koning's volumetric method.

Phosphatase

Kay and Graham Phosphatase Test.[128] As a means of determining whether pasteurization has been properly performed, the phosphatase test has been widely endorsed and is believed to be more reliable than the formaldehyde methylene blue test.

I. Gilcreas Modification.[129] Gilcreas substituted for the Lovibond color slides permanent color solutions.

Reagents. *Buffer Substrate.* Dissolve 1.09 g. of disodium phenyl phosphate ($Na_2C_6H_5PO_4$) and 11.54 g. of sodium veronal (sodium diethyl barbiturate) in water saturated with chloroform and dilute to 1 liter. Add 10 ml. of chloroform and store in a refrigerator.

Folin and Ciocalteu Phenol Reagent. Dissolve 100 g. of $Na_2WO_4 \cdot 2H_2O$ and 25 g. of $Na_2MoO_4 \cdot 2H_2O$ in 700 ml. of water in the 1500-ml. flask of a ground-glass joint refluxing assembly, then add 50 ml. of 85% H_3PO_4 and 100 ml. of HCl. Reflux gently for 10 hours. Cool and add 150 g. of Li_2SO_4, 50 ml. of water, and 4 to 6 drops of liquid bromine, then remove the excess of bromine by boiling apart from the condenser for 15 minutes. Cool, make up to exactly 1 liter, and filter. Keep this stock solution (which must be golden yellow without a greenish tint) in a refrigerator, protected from dust. As needed, dilute 1 volume of the stock solution with 2 volumes of water.

Filter Paper. Whatman No. 40, 11-cm. diameter is free from interfering substances.

Standards. *Gray Solution.* Dissolve 31.9 g. of $CoCl_2 \cdot 6H_2O$, 67.5 g. of $CuSO_4 \cdot 5H_2O$, and 75 g. of $NiSO_4 \cdot 6H_2O$ in water, add 32 ml. of HCl and 45 ml. of H_2SO_4, and dilute to 500 ml.

Red Solution. Dissolve 119 g. of $CoCl_2 \cdot 6H_2O$ in water, filter, wash, add 25 ml. of HCl to the filtrate, and dilute to 250 ml.

Blue Solution. Dissolve 300 g. of $CuSO_4 \cdot 5H_2O$ in water, add 20 ml. of H_2SO_4, and dilute to 1 liter. If crystals separate when cooled below 20°, warm slightly.

Prepare permanent standards by mixing the volumes indicated in the following table. Although designed for comparison in daylight, they may be used with light deflected from a daylight lamp on an unglazed, opaque, turquoise blue glass plate through a depth of 13 ml. of color solution.

Permanent Standards for Phosphatase Test

Phenol	Color Solution		
	Gray	Red	Blue
mg./0.5 ml.	ml.	ml.	ml.
0.01	0.30	0.106	0.96
0.02	0.40	0.140	1.16
0.03	0.55	0.180	1.65
0.04	0.65	0.216	2.10
0.06	0.92	0.286	3.00
0.09	1.30	0.326	4.40
0.12	1.70	0.360	5.70
0.15	2.50	0.396	7.10

PROCESS. *Charge.* Place 10 ml. of the *buffer substrate* and 0.5 ml. of the milk in each of two 20 x 160 mm. test tubes, one for the actual test, the other for a blank designed to detect the deterioration of reagents and the action of interfering substances.

Incubation. To one test tube, add a few drops of *chloroform,* mix thoroughly by rotation, cover with paper toweling to protect from dust, warm to 37 to 39° in a water bath, and heat in an incubator at 34 to 37° for 18 to 24 hours.

Color Formation. After the incubation of the solution in the first test tube, treat the solutions in each test tube as follows. Add 4.5 ml. of the diluted *Folin and Ciocalteu reagent,* mix, and allow to stand 3 minutes. Filter through dry paper, free from phenol and other interfering substances, and transfer 5 ml. of the filtrate to a test tube 13 mm. in diameter. Add 1 ml. of *14% sodium carbonate solution,* mix well by rotating the tube, and heat in a boiling water bath for 5 minutes. Filter at once, cool, and compare the color of the filtrate with the colors of the permanent standards.

INTERPRETATION. If the phenol value of the blank is greater than 0.02 mg., subtract the excess from the phenol value of the actual determination. If this difference is 0.04 mg., it may generally be concluded that the milk was pasteurized at 143° F. (62° C.) for 3 minutes. A higher value indicates inadequate treatment, increasing with the depth of color.

II. Scharer B.Q.C. Field Modification.[130] The description which follows is in substance as communicated to Gilcreas [131] by Scharer (Dept. Health, New York City).

REAGENTS. *Buffer Substrate.* Wash a quantity of disodium phenyl phosphate ($Na_2C_6H_5PO_4$) with ether until free phenols are removed and dry in a desiccator. Dissolve 1.09 g. of the purified salt in 900 ml. of water saturated with chloroform. To the mixture add 50 ml. of borate buffer solution (28.427 g. of sodium borate added slowly with stirring to 900 ml. of warm water, then mixed with 3.27 g. of NaOH, in strong solution, 2 to 5 N, cooled, and diluted to 1 liter). Dilute to 1 liter, add 10 ml. of chloroform, and store in a refrigerator.

Preparation and purification of a buffer substrate solution may be carried out as follows. Dissolve a crushed buffered substrate tablet in a test tube in 5 ml. of warm water, add 2 drops of B.Q.C. solution, and after allowing to stand 5 minutes for color development extract the indophenol with 2 to 2.5 ml. of n butanol. Remove with a medicine dropper the alcohol layer as soon as it separates and reject. Dilute the phenol-free aqueous solution to 50 ml.

B.Q.C. Solution. Dissolve 0.04 g. of 2,6-dibromoquinone chloroimide ($C_6H_5ONClBr_2$) in 10 ml. of ethanol (not denatured) or methanol and store in a refrigerator. If preferred, dissolve one *B.Q.C. tablet* in 5 ml. of either alcohol.

Neutral Normal Butanol. Add to 100 ml. of normal butanol 0.1 to 0.2 ml. of 0.1 N NaOH or a sufficient amount to produce a pale blue color with bromothymol blue indicator.

Stock Color Standards. Dissolve in and dilute to 1 liter with 1% HCl W/V as follows: 59.59 g. $CoCl_2 \cdot 6H_2O$ (red); 62.43 g. $CuSO_4 \cdot 5H_2O$ (blue); 45.05 g. $FeCl_3 \cdot 6H_2O$ (yellow).

Dilute Color Standards. Prepare standards corresponding to 0.2 and 0.5% of raw milk added to pasteurized milk by diluting to 5-ml. stock color solutions in the following amounts respectively: red 0.4 and 0.2, blue 1.5 and 2.2, and yellow 0.5 and 0.5 ml.

PROCESS. *Color Formation.* To 5 ml. of the *buffered substrate solution,* add 0.5 ml. of the sample and shake for a short time. Incubate 10 minutes in a water bath at 38° or, lacking a water bath, in a vest pocket for a somewhat longer period, then remove from the bath, add 6 drops of *B.Q.C. solution,* and immediately shake thoroughly.

Color Reactions are as follows: properly pasteurized milk, gray or brown; properly pasteurized cream, gray or white; raw milk or cream, intense blue. The shade of blue varies with the degree of improper heat treatment.

Color Extraction. Add to the tube 2 ml. of *neutral normal butanol* and invert slowly at least ten times, then allow the alcohol layer containing any indophenol to separate completely.

Color Comparison. In matching the color against the permanent standard, hold the tubes against an opaque milk-glass plate so as to diffuse the light through both sample and standard.

The appearance of any blue color in the butanol layer is an indication of improper pasteurization (or the addition of as little as 2% of raw milk). In the absence of properly pasteurized milk for use as a control, use boiled milk.

Precaution. Thoroughly wash all utensils and tubes. Do not use a resinous bottle closure.

NOTE. Although 18 A.O.A.C. collaborators found that 2% of raw in pasteurized milk could be detected with certainty, it was recommended to continue the study of the test with the view of further increasing its delicacy.

III. Scharer Laboratory Modification.[132] As recently adopted tentatively by the A.O.A.C.,[133] the test is as follows.

REAGENTS. *Borate Buffer.* Dissolve 28.427 g. of $Na_2B_4O_7 \cdot 10H_2O$ in 900 ml. of water, add 3.27 g. of NaOH (81.75 ml. of 1.0 *N* NaOH), and dilute to 1 liter.

Buffer Substrate. Dissolve 0.5 g. of crystalline disodium phenyl phosphate in 5 ml. of water in a test tube (10 x 100 mm.), add 0.5 ml. of borate buffer, shake well, and add 0.04 ml. (or 2 drops from a dropper delivering 50 drops per milliliter) of phenol reagent, shake well, and allow 5 minutes for color development. Extract the indophenol by shaking with 2 ml. of neutral *n*-butanol and allow to stand until the alcohol separates completely. Remove the supernatant alcohol with a pipet or medicine dropper and discard. Dilute the remainder with 100 ml. of borate buffer and sufficient water to make 1 liter.

The solution is phenol-free with a *p*H of about 9.6 (blue to 0.04% thymolphthalein solution in 50% ethanol). Prepare only as needed, avoid contact with rubber, and store in a refrigerator. A darkening means decomposition.

Gibbs Phenol Reagent. Dissolve 40 mg. of 2,6 dibromoquinone-chloroimide in 10 ml. of methanol or ethanol. Keep tightly stoppered and under refrigeration.

Lead Acetate Solution. Dissolve 50 g. of $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ in 100 ml. of water.

Sodium Pyrophosphate Solution. Dissolve 10 g. of $Na_4P_2O_7 \cdot 10H_2O$ in 100 ml. of water.

Permanent Color Standards. (1) *Acid Solutions* (in 1% HCl): (*a*) *red,* 0.5 *N* cobalt chloride solution (59.59 g. of $CoCl_2 \cdot 6H_2O$ per liter); (*b*) *blue,* 30% copper sulfate solution (300 g. of $CuSO_4 \cdot 5H_2O$ per liter); (*c*) *yellow,* 0.5 *N* (*M*/6) ferric chloride solution (45.05 g. of $FeCl_3 \cdot 6H_2O$ per liter). Combine the following quantities, diluting each to 5 ml. with water:

	Color Solution		
Units	Blue	Red	Yellow
	ml.	ml.	ml.
1	0.2	0.35	0.5
2	0.5	0.6	0.55
3.5	0.5	0.5	0.5
5	1.0	0.75	0.5
7.5	1.5	0.75	0.5
10	2.0	1.0	0.25

(2) *Alkaline Solutions* (in 2.8% NH_4OH): (*a*) *red,* 1.8 g. of roseo (aquopentamine) cobaltic chloride $[Co(NH_3)_5 \cdot H_2O]Cl_3$ per liter; (*b*) *blue,* 6.24 g. of copper sulfate ($CuSO_4 \cdot 5H_2O$) per liter; (*c*) *yellow,* 0.84 g. of ammonium chromate $[(NH_4)_2CrO_4]$ per liter. Com-

bine the following quantities and dilute to 5 ml. with water:

Units	Color Solution Blue ml.	Red ml.	Yellow ml.
1	0.25	0.5	0.4
2	0.5	0.75	0.5
3.5	0.8	1.0	0.7
5	1.0	0.6	0.55
7.5	1.25	0.75	0.75
10	1.5	0.25	0.5
15	2.5		0.5
20	2.75		0.5
25	3.0		0.5
50	3.75		0.5
100	4.0		0.5
500	4.5		0.5

These color standards are suitable for use in natural or artificial light provided an opal double-fluxed glass or sheet of Plastacele No. C–1605 HH, 0.015 inch thick, is used as a light filter. Use for color standards test tubes similar to those in which the test is made.

PROCESS. *Incubation.* Pipet 1 ml. of milk into a Pyrex test tube (15 x 125 mm.), add 10 ml. of *buffer substrate*, and mix thoroughly. Warm the mixture to about 40° and continue the heating for 1 hour in an incubator or water bath kept at 37 to 45° (optimum 41°).

Phosphatase Destruction. Heat the tube and contents in boiling water for 5 minutes and cool in ice water.

Clarification. Add 0.1 ml. of *lead acetate solution* and immediately shake vigorously. If proteins do not coagulate and separate sharply, add 0.05 ml. additional of the *clarifier*.

Color Formation. Filter, add to 5 ml. of the filtrate in a test tube (6 x 5/8 inch), 0.5 ml. of *borate buffer*, also if turbid a few drops of *pyrophosphate solution*, then add 0.04 ml. of *Gibbs phenol reagent* (2 drops) from the recommended dropper.

Color Comparison. Mix thoroughly by rotating the tube and estimate the color after 15 minutes by comparison with the permanent color standards.

Control. Mix 5 ml. of the *buffer substrate* and 0.04 ml. of the *phenol reagent*, or incubate 10 ml. of the *buffer substrate* with 1 ml. of a boiled milk sample, proceeding as in the actual test. The amount of indophenol formed in 15 minutes indicates the extent of substrate decomposition.

To 9 ml. of water, add 1 ml. of *borate buffer* and 1 ml. of the milk sample. Mix well and heat in boiling water for 5 minutes as above. The appearance of a blue color indicates to what extent interfering substances in the milk sample influence the result.

INTERPRETATION. A phenol value of 2 units or greater in commercially pasteurized milk indicates inadequate heat treatment.

CREAM NUMBER

Mašek Creamometric India Ink Method.[134]

REAGENT. *India Ink Solution.* Mix 1 part of India ink with 10 parts of water.

PROCESS. Place 0.1 ml. of the *India ink solution* in a graduated cylinder, add water to the 5-ml. mark, then 5 ml. of the milk, invert several times, and let stand at room temperature for 2 hours.

Reading. Measure the thickness of the cream layer to tenths of a milliliter. Multiply the reading by 2 to obtain the cream number.

INTERPRETATION. A cream number of 1 to 4 indicates that the milk has been pasteurized and high numbers that it has not. Flash heating to 73° gives readings like raw milk, but flash heating to 75° reduces the cream number to that of pasteurized milk. Boiled milk gives a cream number of zero. The test is not suitable for milk from old milking cows containing 9 to 11% of fat-free solids.

COLORS

See also Part I, C11 and C12.

The excuse of popular demand, so often

made for the coloring of butter, cannot consistently be made for the coloring of milk, especially as it is practiced usually when the milk is so poor in fat content as to deserve well the name blue milk.

Lythgoe Color Scheme.[135] Lythgoe gives the following scheme for the colors found by him and associates in the examination of 48,000 samples sold in the state of Massachusetts from 1894 to 1902 inclusive. It does not include oil-soluble coal-tar colors that at present would pass into the ether-soluble fraction.

Heat 150 ml. of the milk with *acetic acid* in a casserole. Collect the curd into a mass, strain or pour off the whey, macerate with *ether* in a corked flask, and pour off the ether.

A. *Ether Extract.* Evaporate off the ether on a water bath, add *sodium hydroxide solution* to alkaline reaction, and filter on a wet paper. Wash off the fat and dry the paper which, if annatto is present, is colored orange. Confirm by the stannic chloride test.

B. *Extracted Curd.* (1) If the curd is colorless, no foreign color is present in this fraction.

(2) If the curd is orange or brownish, a coal-tar color or caramel is present; shake with *hydrochloric acid.*

(*a*) If the solution *gradually* turns blue, caramel is indicated. Confirm by tests on whey from original milk.

(*b*) If the orange curd *immediately* turns pink, an azo coal-tar color is indicated. Apply suitable tests.

Formaldehyde

See also Part I, C13.

The value of formaldehyde as a preservative of scientific material has long been recognized. The writers have a specimen of nutmeg fruit enclosing nut, leaf, and stem in a 4% solution (1 to 10 of commercial 40% formaldehyde) prepared nearly forty-five years ago that still retains the natural colors. Succulent fruits and vegetables prepared in like manner ten years ago bid fair to keep indefinitely. Such a high concentration is not, however, necessary, as the history of the dairy industry attests.

Milk containing 0.01% of formaldehyde will ordinarily remain sweet nearly a week and milk of much smaller content will keep for a day or two as well as untreated milk stored in the refrigerator. Naturally such remarkable preserving action appealed to the vendor in his cupidity and the consumer in his ignorance, but not to the nutritionist who recognized that prevention of bacterial action, of itself most desirable, is more than offset by the physiological action of so potent a chemical.

Hehner Iron-Sulfuric Acid Test.[136] To a few milliliters of the milk contained in a test tube, held at an angle, add commercial *oil of vitriol* or *sulfuric acid* to which a trace of *ferric salt* has been added, in such a manner that the acid runs down the side. The appearance of a violet or purple color under the milk in the zone between the two liquids is indicative of formaldehyde. The test may be combined with the determination of fat by the Babcock test.

Note. According to Monier-Williams,[137] a preparation known as *Mystin*, consisting of a solution containing about *10% of sodium nitrite* and *0.3% of formaldehyde*, is sold in England as a milk preservative. Because of the nitrite, the Hehner and doubtless other tests fail. If, however, 5 ml. of a sample, which a test by the Griess-Ilosvay method shows to contain nitrites, is first heated in a boiling water bath for 2 minutes with 0.05 g. of *urea* and 1 ml. of *N sulfuric acid*, the disturbing action of the nitrite is overcome and the usual test applied to the cooled solution is then operative.

Leach Test.[138] This test, which Richmond credits to Leonard and Smith, differs from the Hehner test chiefly in the kind of acid and the manipulation. It has been widely

endorsed in Germany and England as well as in the United States.

Heat slowly with rotation over a full flame a mixture of 10 ml. of milk and 10 ml. of *hydrochloric acid* containing 2 ml. of *10% ferric chloride* per liter. The presence of formaldehyde is indicated by the formation of a violet coloration that persists for several minutes in a carefully conducted test, its absence by a brown color.

Rimini Phenylhydrazine–Nitroprusside Test.[139] To 5 ml. of the milk (or for greater delicacy, 5 ml. of the 20-ml. distillate from 200 ml. of milk acidified with *citric acid*), add 1 ml. of a *4% phenylhydrazine hydrochloride solution*, 3 to 4 drops of freshly prepared *0.5% sodium nitroprusside solution*, and 8 to 12 drops of *10% sodium hydroxide solution*. The formation of a distinct green or blue coloration is indicative of formaldehyde. If acetaldehyde or benzaldehyde is also present, the color will be yellowish or yellow-green, but if these aldehydes are present and formaldehyde is not, the coloration will be red or brown.

Arnold and Mentzel Phenylhydrazine-Ferric Chloride Test.[140] To the distillate prepared as for the foregoing test, or 5 ml. of a solution obtained by filtering a mixture of equal parts of the sample and *ethanol*, add 0.03 g. of *phenylhydrazine hydrochloride* and 4 to 5 drops of *1% ferric chloride solution*, then add to the mixture, with shaking and cooling in water, 12 drops of *sulfuric acid*. A red color appears if formaldehyde is present.

Boric Acid

See also Part I, C13.

Although the opinion of scientists differs as to the culpability of adding to foods boric acid, whether as the free acid or as borax, the consensus of opinion is now against the use of this or any other preservative in milk. During the last quarter of the last century the practice was widespread, as was also lack of dairy sanitation, the latter inviting the former. Fortunately detection is easy.

Turmeric Test. Thoroughly mix 5 ml. of the milk with 7 drops of *hydrochloric acid*, moisten a strip of delicate *turmeric paper* with the mixture, and dry on a watch glass over a boiling water bath. A brick red coloration, changing to blue-black with *ammonium hydroxide*, indicates boric acid free or combined.

Chlorine

Several values, designed by European dairy chemists to test the purity or wholesomeness of milk, involve chlorine or sodium chloride equivalent to chlorine as a factor.

Mathieu and Ferré[141] proposed the *simplified molecular constant* (C.m.S.) obtained by the following formula:

$$\text{C.m.S.} = (F + 11.9S) \times 1.07$$

in which F is grams per liter of lactose found by analysis, S is grams per liter of isotonic hydrated lactose equivalent to chloride calculated as sodium chloride, and 1.07 is the correction factor for the volume of protein plus fat. The range for pure milk of salable quality found by them is 70 to 89 C.m.S.

The *refraction–chlorine value* (B.Cl.Z.), proposed by Beckel,[142] is obtained by the following formula:

$$\text{B.Cl.Z.} = n + 0.06(\text{Cl} - 100)$$

in which n is refractive index of the copper serum and Cl is milligrams per 100 g. of chlorine in the milk. The same author introduced the *chlorine-milk value* (Cl.M.W.), obtained by adding specific gravity, per cent of fat, and 0.1 times the per cent of chlorine.

The *chlorine-lactose value*, proposed by Koestler,[143] is per cent of chlorine divided by per cent of lactose and multiplied by 100. It is designed to detect the abnormalities due to sickness of the cow.

Volhard-Drost Method.[144] Since ashing of milk alone is known to cause a loss of chlo-

rine, Drost devised the two following wet procedures.

A. Weigh 25 g. of milk into a 250-ml. volumetric flask, add 12.5 ml. of *5 N nitric acid*, make up to volume, shake, allow to stand overnight, and filter. Determine chlorine in 50 ml. by the Volhard method (Part I, C8*a*).

B. Substitute for the nitric acid in A 10 ml. of *Fehling copper sulfate solution* (34.639 g. dissolved in water and diluted to 500 ml.) and 17.6 ml. of *0.25 N sodium hydroxide solution*. Make a blank determination, using the reagents only and introduce a correction.

Mohr-Weiss Method.[145] Add to 20 ml. of diluted milk in a 200-ml. volumetric flask 10 ml. of *20% aluminum sulfate* and about 8 ml. of *2 N sodium hydroxide solution*, the exact amount being determined by a blank titration using *azolitmus paper* as indicator. Dilute to the mark, mix, and filter through a dry paper. Determine the chlorine in 100 ml. of the clear filtrate by titration with standard *N/35.5 silver nitrate solution*, using 1 ml. of *10% potassium chromate solution* as indicator.

Alkali Carbonates

Neutralization of soured milk with sodium carbonate or bicarbonate is sometimes practiced. It is detected by the increase in ash and carbon dioxide in the ash, also by several qualitative tests.

Süss Alizarin Test.[146] Shake 100 ml. of milk with 5 to 10 ml. of *0.2% alizarin solution* in *90% ethanol*. If alkali carbonate is present, the solution takes on a distinct rose color, whereas pure milk becomes yellow.

Schmidt Rosolic Acid Test.[147] This test is believed to be less decisive than the Süss test from which it differs in that *rosolic acid* is substituted for alizarin. The color with neutralized milk is rose red, with pure milk brown-yellow.

Eble and Pfeiffer Methanol Test.[148] Shake vigorously three times 20 ml. of milk with 30 ml. of *pure methanol*. If the milk is pure, the curd is in coarse flocks and settles promptly, whereas if the milk has been neutralized it is nearly homogenous and the curd is finely divided, rendering filtration difficult.

Hydrogen Ion Concentration. The *p*H of the serum of pure milk is 6.3 to 6.5, but of neutralized milk it may reach 6.5 or over.

Calcium Sucrate

(Viscogen)

Babcock, who first proposed calcium sucrate as a thickener for milk or cream, also warned against its fraudulent use. It is detected chemically by the high ash and calcium content, the high alkalinity of the ash, and the presence of sucrose.

Ash, Ash Alkalinity, Calcium Oxide. These must be considered in relation to the content of normal constituents of the fat-free solids. Maximum limits for these in pure milk and cream have been established, hence any considerable excess over these limits furnishes circumstantial evidence that a lime salt has been added.

Lythgoe and Marsh[149] found that the maximum per cent of calcium oxide (C), corresponding to the per cent of fat in cream (F), between 15 and 54% is represented by the following equation:

$$C = 0.181 - 0.00246(F - 15)$$

On this basis the calcium oxide in skim milk would be 0.21% and in whole milk intermediate, depending on the fat content.

Sucrose. **Baier and Neumann Molybdate Test.**[150] Process. Add to 25 ml. of the sample in a small Erlenmeyer flask 10 ml. of *5% uranium acetate solution*, shake, and after 5 minutes filter. Shake 10 ml. of the clear filtrate with 2 ml. of *saturated ammonium molybdate solution* and 8 ml. of *5% hydrochloric acid*, and heat in a water bath at 80° for 5 minutes. The appearance of a more or less pronounced blue color, increasing in intensity

with further heating, is indicative of sucrose. A light blue precipitate settles from the deep blue solution on standing stoppered overnight.

Pure milk during 5 minutes heating acquires a light green color which is somewhat more pronounced after 10 minutes, but not the characteristic blue formed with sucrose. On standing overnight the solution is pure green. The test is claimed to detect 0.10% of sucrose.

Lythgoe Modification.[151] PROCESS. Proceed as above, then compare the color of the solution with that formed by adding a few drops of *potassium ferrocyanide* to 1 ml. of *1% ferric chloride solution* in 20 ml. of water. A pale blue color sometimes appears in tests made on pure milk, but this is removed on filtering. Levulose and dextrose also give a blue color, but lactose does not, the intensity of levulose, sucrose, and dextrose being as 10 : 3 : 1, when 1 g. of each sugar is diluted with 35 ml. of water and heated with the *reagent* for 5 minutes.

Rothenfusser Diphenylamine Test.[152] REAGENTS. *Lead Acetate Solution.* Dissolve 500 grams of $Pb(C_2H_3O_2)\cdot 3H_2O$ in 1200 ml. of water.

Diphenylamine Reagent. Mix 10 ml. of 10% diphenylamine solution in ethanol with 25 ml. of glacial acetic acid and 65 ml. of HCl. The solution keeps well.

Fehling Reagents. See Soxhlet Method above.

PROCESS. *Clarification.* To 45 ml. of the sample heated to 85 to 90°, add 45 ml. of *ammoniacal lead acetate solution,* prepared as needed by mixing 1 part of normal lead acetate solution with 1 volume of 15% (for milk and cream with less than 20% of fat) or 2 volumes of 8% (for cream with over 20% of fat) *ammonium hydroxide.* Shake for 15 to 30 seconds and, after 3 to 5 minutes, filter.

Diphenylamine Reaction. Mix 3 ml. of the clear filtrate with 3 ml. of *diphenylamine reagent* in a test tube and heat in a boiling water bath for 10 minutes. If sucrose is present, in 1 or 2 minutes a blue color appears that becomes more intense after 5 minutes. Heating longer than 10 minutes should be avoided.

Fehling Reaction. As a confirmatory test, mix another portion of the clarified filtrate with an equal volume of mixed *Fehling reagents* and heat in the boiling water bath together with the *mixture for the diphenylamine test.* If the precipitation with lead acetate was properly conducted, no copper reduction takes place.

Viscosity. **Pyne Method.**[153] Add to the viscogenized sample *ammonium oxalate* sufficient to precipitate the lime from its gelatinous union with sucrose. Determine the viscosity and compare with that of the sample before treatment and other samples known to be free from viscogen.

VITAMIN A AND CAROTENE

See also Part I, C10.

Olson, Hegsted, and Peterson Spectrophotometric Method.[154] The method originated at the University of Wisconsin.

APPARATUS. *Spectrophotometer.*

PROCESS. *Extraction.* To 100 ml. of the sample, contained in a separatory funnel, add 15 ml. of *ammonium hydroxide,* 100 ml. of *aldehyde-free ethanol,* 75 ml. of *peroxide-free ether,* and 25 ml. of *naphtha* (b.p. 60 to 70°), shaking vigorously after each addition. Allow to stand 20 minutes, draw off the lower layer, and extract with 25 ml. of *peroxide-free ether* plus 10 ml. of *naphtha.* Combine the extracts and after allowing to stand 30 minutes draw off and reject the deposit of solid matter. Transfer the extract to a flask provided with separatory funnel and place on a water bath kept at 70°. Distil off the solvent under reduced pressure and without releasing the vacuum run in 10 ml. of *ethanol* through the separatory funnel. Transfer the liquid to a flask, rinsing with 35 ml. of *etha-*

nol, add 2.5 g. of *potassium hydroxide*, and saponify the fat by refluxing in an atmosphere of *nitrogen* for 20 minutes, then add 100 ml. of water, and cool to 4°. Remove the unsaponifiable matter by shaking consecutively with 75, 25, and 25 ml. of *ether*. Combine the extracts in a separatory funnel and wash with two 100-ml. portions of water, shaking gently, and two 50-ml. portions, shaking vigorously. Cool the ether extract on crushed *dry ice* to freeze out the water. Filter through cotton and wash with cold *ether*. Distil off the ether under reduced pressure, heating in a water bath at 70° with continuous agitation. Immediately after the last of the ether is removed, but before the residue is dried and without releasing the vacuum, run in through an attached separatory funnel 5 ml. of *aldehyde-free methanol*. Dilute further to 10 ml. with the *methanol* and centrifuge to remove any precipitate.

Carotene Determination. Determine by the spectrophotometer.

Vitamin A Determination. Determine by the Baumann and Steenbock biological method.[155]

VITAMIN A

See also Part I, C10.

Chevallier and Manuel Spectrophotometric Method.[156] APPARATUS. *Spectrophotometer.*

PROCESS. *Extraction.* Digest 25 ml. of the sample and 12 ml. of *60% potassium hydroxide solution* at 55° for 3 days. Shake out with 40 ml. of *peroxide-free ether*, wash the ether solution with water, evaporate the ether, and redissolve the residue in 10 ml. of *ether*.

Determine vitamin A in the ether solution by the Chevallier spectrophotometric method.[157]

THIAMIN

(Vitamin B_1)

Jansen Ferricyanide-Thiochrome Fluorometric Method. See Part I, C10.

Vinet and Meunier Modification.[158] PROCESS. *Clarification.* Remove butter fat and casein by refluxing with *acetic acid* and filtering, then dry and extract the residue with *methanol* for the removal of phosphates and lactose.

Color Formation and Reading. See the original method, and the Meunier and Blancpain Diazo-Thiochrome Colorimeter Method, Part I, C10.

RIBOFLAVIN

(Vitamin B_2 or G)

See also Part I, C10.

Emmerie Permanganate Colorimetric Method.[159] In an earlier method for the determination of flavin in urine, Emmerie (Utrecht University)[160] added lead sulfide as an adsorbent of the vitamin, but with liver and milk this was found unnecessary, his procedure for milk being as given below.

APPARATUS. *Pulfrich Photometer* (Zeiss), with 3-cm. cell and screen 47.

PROCESS. All manipulations must be made in red light or very subdued daylight.

Extraction. To 50 ml. of skimmed milk contained in a 100-ml. volumetric flask, add slowly with stirring 50 ml. of *methanol* and hold at 60° for 15 minutes. Cool to room temperature, add 0.1 ml. of *glacial acetic acid*, and adjust to 100 ml. with *methanol*. After shaking, allow to stand 15 minutes, then filter.

Oxidation. Concentrate *in vacuo* 70 ml. of the filtrate (which is 70% of the total, the volume of casein being ignored) to about 20 ml., add 0.5 ml. of *glacial acetic acid* and 1 ml. of *4% potassium permanganate solution*, and shake. After 0.5 minute add 1 ml. of *3% hydrogen peroxide solution* to remove the excess of permanganate, make up to 25 ml., and filter.

Color Reading. Measure the adsorption of the yellow color in the photometer, using a 3-cm. cell and screen 47 and compare with

readings on a *standard solution* of the vitamin treated in like manner.

Whitnah, Kunerth, and Kramer Trichloroacetic Acid Fluorometric Method.[161] Simplicity and accuracy are combined in this modification (Kansas Agricultural Experiment Station) as applied to milk. In its present form, however, it is not applicable to colostrum.

APPARATUS. *Eveready Fluoray Lamp.*

PROCESS. *Solution.* Add to 10 ml. of milk 15 ml. of *10% trichloroacetic acid* and allow to stand 30 to 60 minutes, then whirl in a centrifuge at 2000 r.c.f. for 5 minutes, and decant the supernatant liquid. Add, to 10 ml. of the acid liquid, *sodium hydroxide solution* to neutrality as indicated by *methyl orange.*

Fluorescence Comparison. Dilute the neutral solution until the fluorescence in the light from an Eveready Fluoray lamp matches that of one of a series of standard riboflavin solutions containing 0.06 to 0.12 γ/ml. The final concentration should not exceed that matching a 0.12 γ/ml. standard solution.

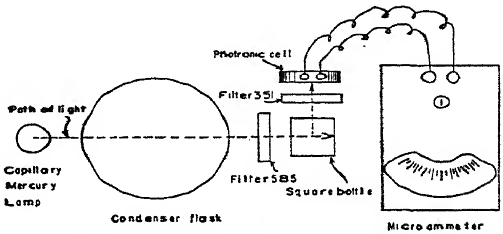
EXAMPLES. In a sample of milk 1.34 γ/ml. of riboflavin were found. In two portions of the same sample, containing added riboflavin, with a calculated content of 2.35 and 3.49 γ/ml. the above authors report 2.12 and 3.19 γ/ml. respectively by the proposed method.

Hand Acetone Fluorometric Method.[162] Working at Cornell University, Hand devised the following method, taking advantage of the possibility that riboflavin is the only green fluorescent substance present in the acetone extract of natural products.

APPARATUS. *Fluorometric Assembly* (Fig. 171), consisting of a capillary mercury lamp (G. E. vertical type H–4 100-watt, nonex envelope), a condenser (1-liter Pyrex flask filled with water), glass filters, 5 cm. square, unpolished (Nos. 585 and 351, Corning), a square bottle in light-tight bottle (A. H. Thomas No. 2248), a photronic cell (Pfalz and Bauer, New York), and a microammeter (full-scale, 15s, 150 ohms internal resistance).

A 25-mm. Corning *Uranium Glass Cube,* conveniently mounted, like that in the assembly for holding the solution, in a square bottle, cut in two just below the shoulder and reunited. A piece of black paper, with a centered opening 1 cm. square, is attached to the surface facing the photocell.

Calibration. Prepare a solution of pure riboflavin, containing 0.1 mg. per ml. in 20%



Courtesy of Ind. Eng. Chem., Anal. Ed. 1939, 11, 307

FIG. 171. Hand Fluorometer Assembly.

ethanol and keep in the dark at 10°. For the calibration of the fluorometer, add to a 66% acetone milk filtrate, from which the riboflavin has been completely removed by exposure to sunlight, amounts of the stock solution ranging from 0 to 2.5 γ/ml. In this way the need of correcting for the riboflavin originally present in the milk is avoided. Take the fluorescence readings and divide by the reading for the uranium glass, thus obtaining the ratio to uranium glass. Plot a curve with 0 to 2.5 γ/ml. as abscissas and ratio to uranium glass from 0 to 2.5 as ordinates.

It is imperative to keep the solutions in the dark and take the readings within 2 or 3 seconds since in 1 minute of exposure to light a 10% reduction of riboflavin takes place.

PROCESS. *Extraction.* Add 50 ml. of *66% acetone* to 25 ml. of milk, mix, and filter through a coarse 15-cm. pleated paper, pouring the first portions of the filtrate through a second time.

Fluorescence Reading. Place the filtrate in the square bottle of the fluorometer, take the fluorescence reading, and determine the ratio of the fluorescence to that of the standard uranium glass.

CALCULATION. Read the concentration from the calibration curve and multiply the reading by the dilution factor 2.83, thus obtaining the result in gammas per milliliter.

The factor 2.83 takes into consideration, first, that 50 ml. of acetone mixed with 25 ml. of milk gives a total volume of 72.3 ml. and, second, that the volume of fat and protein precipitated from 25 ml. of milk is about 1.6 ml. on the basis of 3% by weight of casein and 3.5% of fat, consequently the riboflavin of 25 ml. of milk is contained in 25 × 2.83 = 70.7 ml. of acetone filtrate. The dilution factor for skim milk is 2.87 and for whey 2.89.

Supplee, Bender, and Jensen Clay Adsorbate Method.[163] The method is the final form of the method described in earlier papers by Supplee and associates of the Laboratory of the Borden Company.[164]

APPARATUS. *Fluoray Lamp,* as source of "black light."

Red-Purple Filter No. 587 (Corning Glass Works, Corning, N. Y.).

Corning Filter Screen No. 351, used only for exceptional cases to eliminate blue fluorescence with blue-green radiations down to 4920 Å.

REAGENT. *Standard Riboflavin Solution.* Dissolve 10 mg. of pure riboflavin in 100 ml. of 20% ethanol. Prepare from this stock solution a series containing 0.5 to 0.01 γ/ml. Store in total darkness. Check often for fading or a bluish haze.

PROCESS. *Extraction.* (*a*) *Clay Adsorbates and Similar Residues.* Shake a 2-g. sample with 400 ml. of *80% (by volume) acetone* for 30 to 45 minutes in a dark room, filter, and wash the residue with 10 to 15 ml. of the same *solvent.* Repeat until the eluate shows no more than a faint yellow fluorescence with black light. Transfer the residue to a beaker, add 25 to 30 ml. of water, boil 3 to 5 minutes, cool, and add 112 ml. of *pure acetone.* Transfer the mixture to the original elution flask, agitate for 15 to 20 minutes, filter, and wash with *80% acetone.* Make up eluate and washings to a definite volume.

(*b*) *Fluid and Semi-Fluid Concentrates.* Dilute 2 ml. or 2 g. to 2000 ml. with warm water or *50 to 80% acetone.* If not clear, treat with *80% acetone* as applied to adsorbates.

(*c*) *Whey Products.* Elute 10 g. with 80- to 85-ml. portions of *80% acetone acidified to 0.25 N with sulfuric acid.* Reflux 20 to 30 minutes, decant or filter, and repeat two or three times, finally washing with *neutral 80% acetone.* Neutralize the combined solutions to *litmus* with *sodium hydroxide solution* and make up to volume.

(*d*) *Other Products* (*Dry Yeast, Cereal Products, Oil Seeds, Liver, Meat*). Reflux with *80% acetone* for 2 to 4 hours, after drying at 100° to constant weight. Concentrate the acetone extract, neutralize, and filter.

Fluorescence Reading. Compare the suitably diluted unknown with the *standard* in a totally darkened room, using the Fluoray lamp. Project the beam of ultra-violet radiation directly on the tubes without an intervening screen other than the standard heat-resisting red-purple filter No. 587.

NOTE. Vinet and Meunier,[165] in the solution used for the determination of thiamin (above), also determine riboflavin by electrophotometric comparison of the absorption in the blue with that of pure water.

NICOTINIC ACID

Harris and Raymond Aminoacetophenone-Cyanogen Bromide Colorimetric Method Arnold, Schreffler, and Lipsius Modification. See Part I, C10.

Noll and Jensen [166] endorse the modification.

Ascorbic Acid

(Vitamin C)

See also Part I, C10.

Tillmans-Radeff Indophenol Method.[167] Reagent. *Standard Tillmans Solution.* See Tillmans Method, Part I, C10. Standardize the 0.1 N 2,6-dichlorophenol indophenol against a solution of known vitamin content, fixed in turn against 0.01 N iodine solution.

Process. *Extraction.* Pipet 25 ml. of milk into a 50-ml. volumetric flask, add 5 ml. of *20% thiosalicylic acid solution*, shake, make up to the mark with water, shake again, and filter through a pleated paper, returning the first portions to the paper if not clear.

Titration. Pipet 20 ml. of the filtrate (= 10 ml. of milk) into a porcelain dish, add 0.5 ml. of *saturated sodium acetate solution*, and titrate with *Tillmans solution*, delivered from a 3-ml. micro buret, to a faint rose color within 1 to 2 minutes.

Note. Woessner, Elvehjem, and Schuette [168] note that, in applying the indophenol reaction (see Part I, C10) the interference of certain substances, such as reduced riboflavin, present or formed during the treatment with hydrogen sulfide, may be eliminated by the use of the photoelectric colorimeter for the color measurement.

Reduced Ascorbic Acid

Sharp Modification of the Tillmans Dichlorophenol-Indophenol Method.[169] Reagent. *Standard Dye Solution.* Grind in a mortar 0.2 to 0.3 g. (depending on the purity) of 2,6-dichlorophenol-indophenol, add hot water in 50-ml. successive portions, grinding, decanting and filtering into a 1-liter volumetric flask after each portion until the dye has been dissolved. Cool to room temperature, make up to the mark, and standardize as follows.

Place 5 ml. of a 0.01% solution of pure ascorbic acid in a 200-ml. beaker, add 15 ml. of 0.1 N H_2SO_4 and 15 ml. of water, and titrate without delay to a light pink color that persists for at least 30 seconds. Conduct a blank determination on water plus acid and deduct the number of milliliters required (usually about 0.3 ml.) from that required by the dye.

Divide 0.5 (milligrams of ascorbic acid in the 5-ml. aliquot) by the corrected number of milliliters of dye solution used in the titration and multiply the quotient by 100. The product (F) is the factor for calculating the gammas of vitamin per gram of milk, using 10 ml. in an actual analysis. The amount of dye used in preparing the standard solution should be such that the factor is between 5 and 8.

The standard decreases about 1% daily. Reject the solution after 2 weeks.

Process. Pipet 10 ml. of milk into a 200-ml. beaker, add 25 ml. of *0.1 N sulfuric acid*, and titrate at once with the *dye solution* in subdued light to the same color as in the standardization. The pink color that appears after the first small addition fades after a few seconds. Continue the titration until the color is permanent for several minutes.

Blank. Make a determination on milk allowed to stand until the ascorbic acid disappears and a constant low value is reached.

Calculation. Subtract the result (usually about 4 ml.) of a blank determination and multiply the corrected number of milliliters of dye solution used in the titration by the factor (F) obtained as above directed. The product is gammas per milliliter of ascorbic acid.

Notes on Sampling. Kon and Watson [170] demonstrated that the ascorbic acid of milk when drawn from the cow is in the reduced form, but quickly oxidizes on exposure to air, catalytic metals, light, or heat.

Knight, Dutcher, and Guerrant [171] have devised a simple contrivance whereby the sample may be taken from the cow and kept

without oxidation of the vitamin. It consists essentially of a 500-ml. extra-wide-mouthed ice-jacketed bottle provided with a three-bored stopper through which pass two medium-sized tubes and a third wide tube with a funnel top. One of the medium-sized tubes, provided with a stopcock, serves for the entrance of carbon dioxide, the other, with a Bunsen valve, permits the outward passage of the gas. Slipped over the funnel tube is a pliable rubber tube into which the cow's teat passes, suggesting an adaptor for a Gooch crucible.

Ash

See Part I, C2*f*.

Copper

Callan and Henderson Carbamate Colorimetric Method. See Part I, C8*b*.

Conn, Johnson, Trebler, and Karpenko Modification.[172] The details were developed at the laboratory of the National Dairy Products Corporation, Baltimore, Md.

Apparatus. *Nessler Tubes or Colorimeter.*

Process. *Wet or Dry Combustion.* Dissolve with warming the ash from a 50-ml. portion of milk in 2 ml. of *20% hydrochloric acid,* washing down the sides of the crucible with water. Transfer to a 15-ml. graduated centrifuge tube, keeping the volume down to 7 ml., swirl at 1800 r.p.m. for 10 minutes, decant off the clear liquid, add 2 ml. of water to the residue, centrifuge, and decant. Re-ash the carbon precipitate if it exceeds 0.2 to 0.3 ml., and dissolve.

Sulfide Precipitation. To the combined solution in a centrifuge tube, add dropwise *20% ammonium hydroxide* until a slight precipitate forms, then acidify with 0.5 ml. of *20% hydrochloric acid,* and bring to 10 ml. with water or by evaporation. Heat in boiling water, run in a stream of washed *hydrogen sulfide gas* during 15 minutes of cooling, the last 5 minutes while in a cold water bath. Rinse the delivery tube with 0.5 to 1 ml. of *hydrogen sulfide water containing 1% of hydrochloric acid,* stopper, allow to stand overnight, and centrifuge for 30 minutes at 1800 r.p.m. Decant, wash with 2 ml. of the *sulfide liquid* by centrifuging for 30 minutes and decanting, and dissolve the copper sulfide by heating in a boiling water bath for 10 minutes with 4 drops of *fuming nitric acid.* Cool, dilute to 5 ml., add 1 ml. of *20% ammonium hydroxide,* cool, make up to 10 ml., swirl for 10 minutes, and transfer to another tube.

Color Formation. Add to an aliquot, containing 0.001 to 0.005 mg. of copper in a 15-ml. centrifuge tube, 2 ml. of *ammonium hydroxide,* then dilute to 10 ml. and mix with 1 ml. of *0.1% sodium diethyldithiocarbamate solution.*

Color Comparison. Use for the comparison a freshly prepared *standard copper sulfate solution* (0.01 mg. per ml.) in a colorimeter or a series of standards covering the above range and compare immediately.

Examples. Eighteen samples of raw milk 0.051 to 0.132, average 0.077; 7 samples of pasteurized milk 0.088 to 0.741; 5 samples of dried milk 1.37 to 17.15 γ/g., the higher percentages indicating contamination from equipment. Electrolytic separation gave results agreeing with those of the sulfide separation.

Iron

Herapath Thiocyanate Colorimetric Method. See Part I, C8*b*.

Stugart Modification.[173] Stugart, of Reed and Carnrick, Jersey City, states that the iron content of milk cannot be determined with sufficient accuracy by gravimetric or volumetric methods and that colorimetric comparison of aqueous solutions is not permissible because of fading.

Apparatus. *Colorimeter.*

Reagent. *Standard Iron Solution.* Dissolve 0.5 g. of pure iron wire in 20% HCl with

aid of 1 ml. of HNO_3. Evaporate to dryness, dissolve the ferric chloride in HCl, avoiding a large excess, and dilute to 100 ml.; 1 ml. = 5 mg. of iron. Dilute 1 ml. of the last to 100 ml.; 1 ml. of this final standard = 0.05 mg. of iron.

PROCESS. *Incineration.* Evaporate 100 ml. of the milk to dryness in a platinum dish (or weigh 10 g. of milk powder), dry at 110°, slowly carbonize in an open muffle furnace, close gradually, and allow to remain overnight at just below dull redness.

Solution. Take up in a little *dilute iron-free hydrochloric acid,* filter, ignite the filter paper and unburned carbon, again take up in dilute acid, filter, and add the filtrate to the original filtrate, then make up to 50 ml. Remove an aliquot, containing 0.025 to 0.10 mg. of iron, to a 150-ml. beaker, add 5 ml. of *hydrochloric acid,* dilute to about 25 ml., boil 20 minutes, cool, add 1 drop of *nitric acid,* and make up to 25 ml. in a volumetric flask.

Color Formation. Pipet 10 ml. into a 30- or 50-ml. separatory funnel, add 1 ml. of *iron-free, redistilled hydrochloric acid* and enough *0.1 N potassium permanganate solution* (usually 1 or 2 drops) to produce a pink color which persists 20 seconds. Add from pipets exactly 5 ml. each of *isoamyl alcohol* and *20% potassium thiocyanate solution,* shake vigorously for 30 seconds, allow to separate, and draw off the aqueous layer.

Color Comparison. Place 1 and 2 ml. of the *final standard,* equivalent respectively to 0.05 and 0.10 mg. of iron, into two 50-ml. volumetric flasks, add to each 1 drop of *nitric acid* and 10 ml. of *hydrochloric acid,* and dilute to volume. Transfer 10 ml. of each to 30- or 50-ml. separatory funnels and proceed as above directed. Compare the unknown with the *standards* in the colorimeter.

Lyons Thioglycolic Acid Colorimetric Method. See Part I, C8*b*.

Leavell and Ellis Modification.[174] This modification was developed in the Bureau of Animal Industry, Washington.

APPARATUS. *Color Comparison Tubes.*

REAGENTS. *Thioglycolic Acid Reagent.* Add 4 ml. of the acid to a solution of 8 ml. of NH_4OH in 50 ml. of water.

Standard Iron Solutions. Dissolve 1 g. of iron wire in dilute H_2SO_4 and oxidize with HNO_3. Expel oxides of nitrogen and excess of HNO_3 by boiling and dilute to 1 liter. Prepare by dilution other standard solutions as needed, adding extra H_2SO_4 to prevent hydrolysis of the dissolved iron salts to insoluble basic compounds.

PROCESS. *Digestion.* Heat to boiling over an electric plate 5 ml. of milk, or 0.5 g. of dried milk, with 3 ml. of *sulfuric acid* and 0.5 ml. of *perchloric acid* in a micro Kjeldahl flask, add a second portion of 0.5 ml. of *perchloric acid* to the cooled flask after charring is marked and two others later during the digestion (30 minutes to 2 hours).

Color Formation. When cool, transfer the digest to a 150-ml. beaker, rinse with small portions of water, and adjust at first with *ammonium hydroxide* to slight alkalinity to *litmus paper,* then to distinct acidity with *sulfuric acid,* adding 5 to 10 drops beyond neutrality. Add 1 ml. of *thioglycolic acid reagent,* then 1 to 2 ml. of *ammonium hydroxide* to strong alkalinity.

Color Comparison. Match the color in 12 x 20 mm. tubes against the *standard solutions.*

EXAMPLES. Cow's milk, 3 samples, 0.06 to 0.075, goat's milk, 7 samples, 0.006 to 0.08, and dried milk, 4 samples, 1.00 to 1.10 mg. per 100 ml. or g.

2. EVAPORATED MILK

Since in the manufacture of unsweetened evaporated milk whole milk is reduced to half its volume without loss of solid constituents, a fluid prepared by mixing the commercial product with an equal volume of water has approximately the same general composition as the original milk. Paradoxically,

however, one constituent, namely fat, is not completely extracted from the dried milk when subjected to continuous extraction by the method long regarded as standard for milk. This is claimed to be due to the coagulation of the protein as particles that surround the minute fat globules and protect them from the solvent. Other constituents are accurately determined in the diluted product by the usual methods designed for milk.

Sample

Thoroughly mix the contents of the can, making sure that any sediment is incorporated or any separated fat is emulsified. Weigh 50 g. in a small beaker or sugar dish and transfer to a 100-ml. volumetric flask, using a little hot water for rinsing. Make up to the mark, mix, and without delay remove with a pipet aliquots for the various determinations.

The entire contents of the usual 14.5-ounce can may be made up to 1 liter; weigh the can and contents at the start and the rinsed and dried can after removal of the contents. This procedure permits determination of the net contents but involves more calculation and leaves no reserve for subsequent work.

Solids; Protein; Lactose; Ash

Use the same volumes of the diluted sample and follow the same methods as given for Milk. Multiply the results by 2.

Fat

Röse-Gottlieb-Patrick Method. Use 10 ml. of the diluted sample and proceed as directed under Milk. Multiply the result by 2 to correct for the dilution.

Hunziker and Spitzer Modification of the Babcock Centrifugal Method.[175] Process. Weigh into an 8 or 10% Babcock milk bottle 9 g. of the diluted sample, prepared as above described, together with 9 ml. of water. Add 17.5 ml. of *sulfuric acid* of the usual Babcock strength, shake until the curd disappears, and whirl for 5 minutes. Fill to the neck with hot *1 + 1 sulfuric acid* and whirl again. If the sulfuric acid is diluted just before using, the heat generated supplies sufficient warmth. Fill to the top of the graduation with hot water, whirl a third time, and read while warm.

Calculation. Multiply the reading by 2.

3. SWEETENED CONDENSED MILK

Prepare the sample and determine **solids, protein, lactose,** and **ash** as directed under Evaporated Milk, but dilute 40 g. instead of 50 g. to 100 ml. and multiply the results by 2.5 instead of 2.

Fat

Röse-Gottlieb-Patrick Method. Use 10 ml. of the diluted solution (see above) and follow the instructions given under Milk. Multiply the results by 2.5 to correct for the dilution.

Babcock-Farrington Centrifugal Method.[176] Process. *First Acid Treatment.* Pipet 17.6 ml. of the diluted milk into a 10% Babcock milk bottle. Add 3 ml. of *sulfuric acid* of the strength used in the Babcock test and shake vigorously, thus coagulating the protein and trapping the fat so that the sugars may be removed subsequently in the solution. Whirl at high speed in a steam turbine machine or one otherwise heated to about 200° F. (93° C.).

Second Acid Treatment. Remove from the centrifuge and cautiously pour off the liquid, avoiding breaking the lump of curd. Add 10 ml. of water to the curd, shake, then add 3 ml. of *sulfuric acid,* whirl, and decant as before.

Third Acid Treatment. Finally add 10 ml. of water and 17.5 ml. of *sulfuric acid* and proceed as in the usual Babcock method.

CALCULATION. Multiply the reading by 18 and divide by 7 to obtain the percentage of fat.

CITRIC ACID

Hartmann and Hillig Method. See Milk above.

Arup Modification.[177] The following modification has been successfully used at the Butter Testing Station, Dublin.

PROCESS. *Clarification.* To 20 g. of the sample in a wide-neck 100-ml. volumetric flask, add 60 ml. of hot water, heat on a boiling water bath, and thoroughly mix, then add 8 ml. of *50% trichloroacetic acid*, mix, and heat as before for 30 minutes with occasional shaking. Cool, make up to the mark, filter, and transfer a suitable aliquot (usually 75 ml.) to a 100-ml. beaker.

Lead Citrate Precipitation. Add *phenolphthalein indicator* and *sodium hydroxide solution* (at first strong, then 0.1 N) sufficient to produce a faint pink color. Add an excess (usually 10 ml.) of *15% normal lead acetate solution*, mix, allow to settle, and filter on a Gooch crucible prepared with 0.1 to 0.2 g. of asbestos. Suck dry and wash with 10 ml. of water. It is not necessary to remove all the sugar or wash the last traces of the precipitate onto the filter.

Lead Removal. Transfer the crucible together with the asbestos and precipitate to the beaker previously used. Add 70 ml. of hot water and 15 ml. of *50% (by volume) sulfuric acid*, stir, warm on a water bath 15 minutes with occasional stirring, and transfer to a 110-ml. volumetric flask. Cool, make up to the mark with water, filter, pipet 100 ml. into a 250-ml. Erlenmeyer flask, and proceed as directed under the Hartmann and Hillig Method, except that the *potassium permanganate solution* is added slowly with stirring, instead of all at once, to the acidified citric acid solution containing potassium bromide.

CALCULATION. Obtain the correction for the volume of the first precipitate by the following formula:

in which V is the volume of the precipitate in milliliters, W is the weight of the sample in grams, and F and P are the percentages respectively of fat and protein ($N \times 6.38$) in the sample.

Assume 0.4 ml. as the correction for the lead sulfate precipitate and asbestos.

EXAMPLES. *Fresh Milk*, 25 samples, 0.150 to 0.206, average 0.168; *Condensed Milk*, full cream, unsweetened, 5 samples, 0.400 to 0.494; *Condensed Milk*, full cream, sweetened, 5 samples, 0.458 to 0.506, separated, sweetened, 6 samples, 0.468 to 0.588; *Sterilized Tin Cream* (22 to 25% fat), 4 samples, 0.133 to 0.166; *Milk Powder, Spray Process*, 6 samples, 1.62 to 1.86; *Roller Process*, 5 samples, 1.73 to 1.88% of anhydrous citric acid.

SUCROSE

Knight and Formanek Polariscopic Method.[178] After due experimentation, Knight and Formanek, of the U. S. Customs Service, Port of New York, combined the use of phosphotungstic acid as a clarifier, as proposed by Richmond,[179] with the double dilution procedure of Wiley and Ewell.[180]

APPARATUS. *Bates Polariscope.* Instrument adjusted for maximum accuracy.

Bichromate Cell.

PROCESS. *Solution.* Dry the outside of the unopened can and weigh together with contents (usually about 14.5 ounces), remove the contents to a 500-ml. volumetric flask, rinsing with hot water, dry the can, and deduct its weight from that of the can plus contents. Shake to dissolve sugar crystals, fill to mark, cool to room temperature, and

shake again. Pipet 50- and 100-ml. aliquots into 200-ml. volumetric flasks.

Clarification. Add to each aliquot for each 10 g. of the sample in the aliquot 1.7 ml. of *5% phosphotungstic acid solution* and 2.1 ml. of *25% normal lead acetate solution*, shaking after each addition. Make up to volume, shake, and filter through a dry paper.

Deleading. To the filtrates, add *potassium oxalate crystals* in 0.1-g. portions with constant shaking until a curdy precipitate forms that settles quickly, leaving a clear supernatant liquid. Filter through a hardened filter containing 3 to 5 g. of fuller's earth, testing the first 10-ml. portions of each filtrate with *potassium oxalate crystals.*

Direct Polarization. Polarize in a 200-mm. tube at 20° with great care, since otherwise the errors of double dilution will be considerable. Multiply the reading of the dilute solution by 4 and subtract from the product the reading of the stronger solution. The difference is the direct polarization (P) corrected for the volume of the precipitate.

Invert Polarization. Pipet a 50-ml. aliquot of each filtrate into a 100-ml. volumetric flask, add 5 ml. of *hydrochloric acid* (38.8%), and allow to stand overnight at room temperature (not below 20°) for inversion. In the morning, add a few drops of *phenolphthalein solution,* then *strong sodium hydroxide solution* to a pink color. Remove the pink color by cautious addition of *0.1 N hydrochloric acid,* cool to room temperature, make up to the mark, and polarize, preferably using 400-mm. tubes for an instrument other than that of the Bates type. Obtain the corrected invert polarization by subtracting the polarization of the stronger solution from four times that of the weaker. Multiply this corrected invert polarization by 2 (except where 400-mm. tubes were used). The product P' is the invert polarization corresponding to the direct polarization (P).

Calculation. Obtain the percentage of sucrose (S) in the sample (W) from the above data and the temperature of invert polarization (T) by the following formula:

$$S = \frac{26{,}000(P - P')}{W(141.7 - 0.5T)}$$

4. MILK POWDER

Without considering in detail the various processes for drying milk, it may be stated that the products differ widely in physical characters. The powder made by the Spray process consists of hollow globules which, as seen under the microscope, suggest diminutive Christmas tree ornaments. If these globules are broken by grinding, as sometimes practiced by some manufacturers, the microscopic characters are markedly changed. Drum process powder has certain physical characteristics dependent on the formation of crusts which are not readily described, but are recognized after some experience.

Skim milk powder is the more common product and its analysis presents fewer difficulties than that of whole milk powder.

Sample

Rub up 6 (or 60) g. of a whole milk powder, or 4 (or 40) g. of a skim milk powder with cold water in successive portions, decanting after each into a 100- (or 1000-) ml. volumetric flask. Make up to the mark, shake well, and, without delay, remove suitable aliquots with a pipet as in the analysis of evaporated milk.

Analysis

Follow the details of analysis given under Evaporated Milk, which with few exceptions are essentially those for whole milk.

In the analysis of some powders, it is preferable to weigh out individual charges as recommended in the *A.O.A.C. Methods.* If the fat is not evenly distributed, large por-

tions or even the whole sample may be used, depending on aliquots to reduce the portions for analysis.

Riboflavin

Sullivan and Norris Photometric Method.[181] The method originated in the laboratory of Cornell University.

Apparatus. *Sullivan and Norris Special Photoelectric Photometer.* Details of construction are given in the paper; also the method of calibration.

Reagents. *Acid-Acetone Reagent.* Dilute 750 ml. of acetone to 1 liter with 1.0 *N* H_2SO_4.

Sodium Hyposulfite Reagent: 5% $Na_2S_2O_3 \cdot 5H_2O$ solution in 2% $NaHCO_3$ solution.

Process. *Extraction and Decolorization.* Place 15 g. of the sample in a 250-ml. Erlenmeyer flask and add 100 ml. of *acid-acetone reagent.* Add 1 to 3 ml. of *30% hydrogen peroxide solution,* depending on the degree of caramelization, and reflux for 20 minutes. Cool before disconnecting, stopper with a paraffined cork, and cool in the refrigerator for 30 minutes.

Neutralization. Neutralize by addition of 25 ml. of *1.0 N sodium hydroxide solution* and make up to 150 ml. with 22 to 24 ml. of a *buffer solution* which for dried whey has a *p*H of 6.8 and for dried skim milk and buttermilk a *p*H of 4.6. Cool the flask again in the refrigerator for 30 minutes, then filter.

Reading. Read the absorption of this 10% solution in the photometer, then completely reduce the riboflavin to the leuco form by addition of 15 to 30 drops of *sodium hyposulfite* reagent and read again.

Calculation. The initial equation is

$$K = \frac{2.3}{C \times D} \log \frac{I_0}{I}$$

in which C is the concentration in moles per liter and D is the thickness of the absorbing layer in centimeters. I_0 and I are respectively the absorption reading with the zero current and in the normal manner.

Since a thickness of 1 cm. is always used and $K = 2.78 \times 10^4$, to obtain the concentration in gammas per milliliter (c), the equation is expressed as follows:

$$c = \frac{2.3 \times 376 \times 10^3}{2.78 \times 10^4 \times 1} \log \frac{I_0}{I} = 31.1 \log \frac{I_0}{I}$$

Obtain two values of c, before (c_1) and after (c_2) reduction, from which derive the concentration of riboflavin in gammas per milliliter (M) by the formula

$$M = 1.307(c_1 - c_2)$$

If I_0 remains constant, a table may be prepared for obtaining M from c.

Ascorbic Acid

Woessner, Elvehjem, and Schuette,[182] in applying the indophenol method for ascorbic acid to roller-process milk powder, mix 145 g. of the sample with 1 liter of copper-free water, and heat at 50°. In the determination of dehydroascorbic acid in milk powder, they proceed as follows. Add a few drops of *dibutyl phthalate* to control foaming and run *hydrogen sulfide* through the liquid for 20 minutes. Immediately remove 25 ml., add 75 ml. of *Willberg reagent,* and shake to break up curd. Remove the hydrogen sulfide by a stream of *oxygen-free nitrogen* (20 minutes), filter, and pipet 5 ml. of the clear filtrate into the colorimeter tube, then add 10 ml. of the *dye-acetate solution,* and read the galvanometer after 15 and 30 seconds.

5. CREAM

Fat

Cream is analyzed in the same manner as milk, using, however, especially for the Babcock test, a smaller charge.

Babcock Sulfuric Acid Centrifugal Method. Apparatus. The *Babcock Pipet* for milk, calibrated with 17.6% of water, delivers practically 18 g. of milk and it is on this basis that

the accuracy of the method has been established. The specific gravity of cream with up to 20% of fat is such that by using an 18-ml. pipet and bottles showing percentages equivalent to those on the milk bottles, but with an extended scale, the method gives fairly accurate results. For higher percentages of fat, cream must be weighed on special balances designed for dairy use. In the chemical laboratory, an analytical balance may be used for an occasional determination.

Cream Test Bottles. In order to furnish capacity for volumes of butter fat exceeding 10% and when using the charge of 18 g., special bottles were devised soon after the introduction of the method. These were especially designed for use in cream-gathering creameries which up to that time had paid for cream by weight regardless of the fat content which often varied as much as 1 : 2.

The *Bartlett Bottle* provided for the extra volume of fat in a bulb blown in the middle of the graduated neck of the test bottle. This was not satisfactory in the hands of unskilled workers who found difficulty in adding the proper amount of water to bring both the top and bottom of the fat column outside the bulb.

Winton Bottle. This bottle differs from the ordinary milk test bottle only in that the neck for about the same length of graduation contains three times the volume of fat and is correspondingly graduated up to 30%. It was introduced into cream-gathering creameries not only in the United States but also in foreign countries.

Other Cream Test Bottles. The marketing of heavy and extra heavy cream called for test bottles of the Winton type with graduations up to 50% or more, necessitating either a neck of wider bore or greater length. At the present time the A.O.A.C. gives specifications for three types of cream test bottles: (1) 50%, 9 g., short-necked, 6-inch; (2) 50%, 9 g., long-necked, 9-inch; and (3) 50%, 18 g., long-necked, 9-inch. The Association also specifies that the sensitivity of the cream-weighing scales should be 30 mg.

Phosphatides

(Phospholipides)

Wiese, Nair, and Fleming Immiscible Solvent Method.[183] The procedure, as conducted at the Borden Research Laboratories, combines the Mojonnier modification of the Röse-Gottlieb method with determination of phosphorus by the Briggs molybdate-hydroquinone sulfite colorimetric method in the fat thus extracted.

Apparatus. *Color Comparison Tubes.*

Reagents. See below.

Process. *Extraction.* Weigh 6 to 7 g. of (40%) cream into a Mojonnier extraction flask, add 1.5 ml. of *ammonium hydroxide*, and extract as directed for the Mojonnier modification with *ethanol*, *ether*, and *naphtha* (see Röse-Gottlieb Method), using 25 ml. each of *ether* and *naphtha* for the first two of the three extractions. Evaporate the solvents, dry the fat to constant weight, and calculate the percentage.

Incineration. Take up the fat in *ether* or *naphtha*, transfer to a platinum or preferably sillimanite evaporating dish, add 2 ml. of *50% ethanolic magnesium nitrate solution*, and evaporate to dryness on a hot plate. To residue add dropwise 1 ml. of *nitric acid*, heat gently over a low flame, increasing gradually the size of the flame until the mass is completely charred, then complete the ashing in a muffle furnace. To remove the last traces of carbon, take up in a little water, evaporate, and again ignite cautiously.

Phosphorus Determination. Take up the ash in just enough *dilute hydrochloric acid* for complete solution, dilute to 20 ml., and determine the phosphorus colorimetrically in 3- to 4-ml. aliquots by the Briggs molybdate-hydroquinone sulfite method [184] as follows. Add 1 ml. each of *5% molybdate solution in 5*

N sulfuric acid, 1% hydroquinone solution containing 1 drop of sulfuric acid, and *20% sodium sulfite solution,* and compare with a *standard solution of monopotassium phosphate* prepared by diluting a stock solution (1 ml. = 1 mg. of P) treated in like manner.

CALCULATION. Express results in terms of milligrams of phosphorus per 100 ml. of cream.

6. ICE CREAM

Originally a frozen mixture of cream with sugar and flavoring, ice cream today is commonly an homogenized mixture containing additional constituents such as milk, skim milk, evaporated milk, sweetened condensed milk, milk powder, and fresh butter, as well as gelatin or other thickener. The commercial advantage of combining evaporated skim milk or skim milk powder with storage butter is that these may be stored during the flush season, whereas milk or cream may not because of their bulk and greater tendency to spoilage.

SAMPLE

1. If the ice cream is homogenous, as it usually is when the flavor was added as an extract (vanilla, lemon, etc.), allow to melt, mix thoroughly, remove 40 g. to a 100-ml. volumetric flask, make up to the mark, remove aliquots, and proceed with the determinations as directed under Sweetened Condensed Milk above.

2. If the ice cream contains solid particles, such as minced nuts, berry seeds, and vegetable tissues, mix the sample with a spoon before it thaws completely and remove 40 g. to a dish or small beaker, then allow to melt, and decant onto a Gooch crucible or similar device without a mat of any kind, collecting the strained liquid in a 100-ml. volumetric flask. Finally transfer the solid matter to the crucible and wash with cold water cautiously, using suction when a mat forms. Suck dry, remove visible moisture with bits of filter paper and weigh the Gooch crucible plus the moist vegetable tissues. Dry the crucible and contents, weigh, and obtain the *dry vegetable tissues.* Make the liquid up to the mark and remove aliquots without delay.

SOLIDS, PROTEIN, LACTOSE, SUCROSE

See directions given under Sweetened Condensed Milk above.

FAT

Röse-Gottlieb-Patrick Method. Proceed according to the details given under Milk, using 10 ml. of the diluted sample.

Babcock-White Centrifugal Method.[185] PROCESS. *Acid Treatment.* Pipet 15 ml. of the diluted sample (equivalent to 6 g. of the original sample) into an 8% Babcock milk bottle, add 3 ml. of water and 4 ml. of *Babcock sulfuric acid,* shake thoroughly, and allow to stand 2 minutes, then add a second portion of 4 ml. of *sulfuric acid,* and shake again.

First Whirling. If the solution is black, repeat the work, using less acid; if brown, centrifuge at high speed for 3 minutes and fill to the neck with water at 65°.

Second Whirling. If any black specks appear in the fat, shake vigorously for a few seconds and whirl a second time for 3 minutes.

Third Whirling. Fill with water to the top of the graduation, whirl a third time for 2 minutes, place in water at 65°, and read after a few minutes.

CALCULATION. Multiply the reading by 3.

Overman and Garrett Non-Acid Centrifugal Method.[186] REAGENTS. (1) Mix 75 ml. of c.p. NH_4OH, 35 ml. of butanol, and 15 ml. of denatured ethanol. (2) Dissolve with heat 200 g. of $Na_3PO_4 \cdot 12H_2O$ and 150 g. of $NaC_2H_3O_2 \cdot 3H_2O$ in 1 liter of water.

PROCESS. *Treatment with Reagents.* Weigh 9 g. of the sample into an 8 or 10% milk test bottle or a special 20% ice cream test bottle,

add exactly 2.5 ml. of *reagent 1* and mix thoroughly, then add 9 to 10 ml. of *reagent 2* and again mix. Heat in a water bath to boiling and continue the heating several minutes thereafter with occasional shaking.

Centrifuging. After the fat separates as a clear layer (15 to 30 minutes), whirl successively 5, 2, and 1 minutes, adding hot water as in the Babcock test.

Reading. Finally place the bottle in water at 130 to 140° F. for 5 minutes and read from the bottom of the lower meniscus to the top of the upper meniscus.

ACCURACY. The results agree closely with those of the Röse-Gottlieb-Patrick method.

LECITHIN PHOSPHORIC ACID

Juckenack-Gronover and Lederle Method.[187] PROCESS. *A. Dry Combustion.* Evaporate 30 to 50 g. of the sample together with 15 to 25 g. of *ground pumice stone* to a soft paste and complete the desiccation by addition of *anhydrous sodium sulfate.* Grind and extract with *absolute ethanol*, evaporate the ethanol from the extract, and burn to an ash with 3 ml. each of *50% magnesium acetate* solution and *8% ethanolic potassium hydroxide solution.* Finally take up in *nitric acid.*

B. Wet Combustion. An alternate procedure is to treat the ethanolic extract with 5 ml. of *sulfuric acid* and oxidize the organic matter by addition of small portions of *nitric acid* with gentle heating, finally heating at a higher temperature until colorless.

Phosphoric Acid Determination. In either case determine phosphoric acid by the Molybdic-Magnesium Pyrophosphate Method (Part I, C8*a*).

OLEOMARGARINE

Whitehead and Dunson Simplified Calculation Method.[188] The method, based on average Reichert-Meissl number (30.96) of butter fat, originated at the University of Georgia.

PROCESS. *Fat Extraction.* Allow the cream to soften, then mix well with an egg beater. To 100 g. of the homogeneous product, in a separatory funnel, add 12.5 ml. of *ammonium hydroxide*, 50 ml. of *ethanol*, 100 ml. of *ether*, and 100 ml. of *naphtha*, shaking for 30 seconds and allowing to stand 20 minutes after each addition. Separate the fat solution as completely as possible and filter, then repeat the extraction with *ether* and *naphtha* and filtration, and draw off the clear solution, washing the filter and tip of the funnel with a mixture of *ether* and *naphtha.* Evaporate the combined extract, and dry the fat at 90° to constant weight.

Saponification. Weigh into a flask 5 g. of the fat at about 40° and saponify for 1 hour on a water bath with 10 ml. of *ethanol* and 2 ml. of *sodium hydroxide solution.* Evaporate the ethanol and cool to room temperature. Dissolve the soap in 100 ml. of freshly boiled water, heat to 60 to 70°, add 40 ml. of *3.15% sulfuric acid* and a few bits of *pumice stone*, then distil 110 ml. in 28 to 32 minutes.

Titration. Use *0.1 N sodium hydroxide solution* and *phenolphthalein indicator* and titrate 100 ml. of the filtered distillate.

CALCULATION. Obtain the Reichert-Meissl number (R) by the formula

$$R \quad \frac{N \times 1.1 \times 5}{G}$$

in which N is the milliliters of standard alkali and G is the grams of sample. Then obtain the percentage of oleomargarine fat (O) and butter fat (B) in the fat by the following formulas:

$$O = \frac{30.96 - R}{0.31}$$

$$B = 100 - O$$

7. BUTTER

Butter is a natural product only in the sense that fat, its chief constituent, is derived

with no material change from milk. If made from ripened cream, it contains small amounts of substances formed by enzymes and bacterial action that contribute desirable flavor at first, but eventually undesirable flavor if care is not taken in storage. Salt is an addition varying according to the amount added and the loss in buttermilk during working.

Proximate Composition. The analyses in the following table do not include small amounts of lactic acid, diacetyl formed from acetylmethylcarbinol during ripening, natural or artificial colors, and numerous trace constituents, some derived from careless handling and others from contact with metallic containers. The composition, also the chemical and physical values of butter fat, are considered under the heading Fat Constants below. Analysis of the butter is primarily designed to determine the fat content, whereas the purpose of analysis of the fat from the butter is to learn the chemical constituents of the fat as a contribution to pure science or the source of the fat, whether or not derived solely from milk, for inspection purposes.

Sample

Draw sample from tubs with the regular gouge-formed trier inserted diagonally from the top edge to the opposite bottom edge so as to secure a perfect core. Cut pound prints crosswise and lengthwise into quarters and take one quarter of each; if there are four quarter-pound subprints, take one from each pound. Transfer to a fruit jar, close tightly, heat in hot water with occasional shaking until melted, taking care that a core does not remain solid, then shake well until uniform, immerse the jar in ice water, and shake continually when approaching the congealing point until the whole mass has solidified.

Moisture, Fat, Curd, and Salt

Wiley Gravimetric Method.[189] **I. Moisture.** Dry about 2 g. of the sample in a tared flat-bottom metal dish about 5 cm. in diameter to constant weight in a boiling water oven. Calculate the loss in weight as moisture.

II. Fat. *A. Indirect Process.* Stir with several portions of *naphtha* the residue from 2 g. of the sample obtained in the determina-

Average Composition of Butter

	Samples	Water	Fat	Curd, etc.	Ash	Authority
		%	%	%	%	
Salted						
United States	645	13.87	82.47	1.15	2.51	Thompson, Shaw, and Norton *
Australia	19,470	13.84	83.50	0.76	1.82	Crowe †.
Unsalted						
United States	242	13.07	85.24	1.57	0.12	Woll ‡
France	108	13.76	84.77	1.38	0.09	Vieth §

* *U. S. Dept. Agr., Bur. Anim. Ind.* 1912, *Bull.* 149. † *J. Dept. Agr. Victoria* 1913, **11,** 357. ‡ Farrington and Woll: *Testing Milk and Its Products,* Madison, 1916, p. 259. § Analyst 1891, **16,** 1.

tion of moisture (above) until the fat is dissolved, collect the residue on a Gooch cruci-

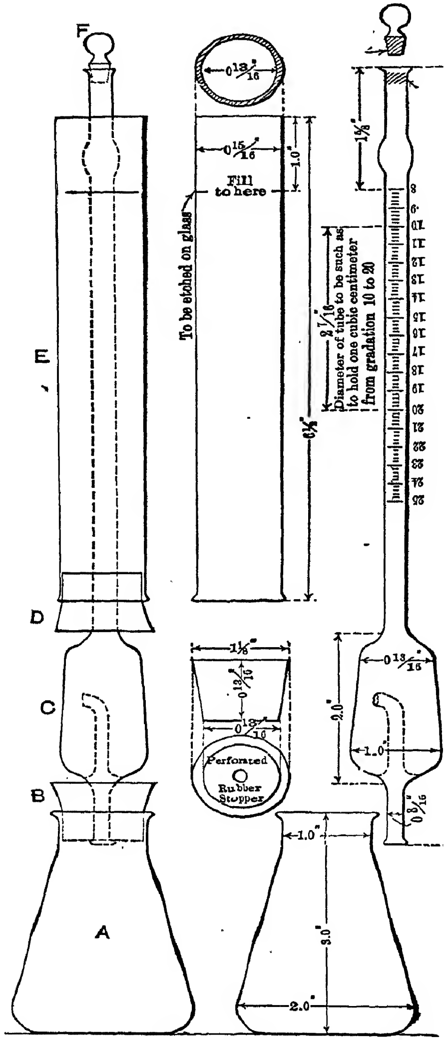


FIG. 172. Gray Moisture Apparatus.

ble, and wash with *naphtha* until all fat is removed. Dry the crucible containing the curd and ash (salt and other mineral matter) in a boiling water bath to constant weight, cool in a desiccator, and weigh.

The weight of the residue before extraction less the weight of curd and ash represents the fat.

B. Direct Process. Evaporate the naphtha containing the dissolved fat obtained as above described, dry the fat in a boiling water oven, and weigh. The former procedure is more convenient and less subject to error.

III. Curd (Protein). Heat cautiously the curd and ash obtained in the indirect determination of fat, as above described, finally raising the heat to dull redness until the curd is destroyed and a white ash remains. Cool in a desiccator and weigh. Obtain the weight of the curd by difference.

IV. Salt. *A. Indirect Process.* Treat the ash contained in the Gooch crucible with hot water until the soluble ash is dissolved and determine the chlorine either gravimetrically or volumetrically by titrating with *standard silver nitrate solution,* using *potassium chromate indicator* (Part I, C8*a*).

$$\text{Weight of AgCl} \times 0.2474 = \text{weight of Cl}$$

$$\text{Weight of AgCl} \times 0.4078 = \text{weight of NaCl}$$

B. Direct Process. Weigh a new portion of 5 to 10 g. of the sample into a counterpoised lipped dish and wash with about 25 ml. of hot water into a separatory funnel. Shake, allow to settle, and draw off the aqueous layer. Add about 25 ml. of hot water to the fat in the separatory funnel, shake and draw off the aqueous layer, repeating the extraction until all chlorine is removed. Determine chlorine in the combined aqueous extracts as directed above.

Moisture

Patrick Approximate Gravimetric Method.[190] This method may be carried out by the layman without recourse to a chemical balance.

Tare on a balance accurate to 0.1 g. an

aluminum beaker together with a glass rod. Add about 10 g. of the butter sample and weigh again. Heat over an alcohol, gas, or colorless kerosene flame, constantly moving to avoid spattering and discolorization of the curd. In about 15 minutes weigh the beaker and the dried butter and determine the loss.

Gray Distillation Volumetric Method.[191] The method is especially designed for the use of inspectors and dairymen without laboratory equipment.

Apparatus. Fig. 172 shows the parts assembled and disconnected. The specially graduated tube *C* has a glass stopper at the top and a safety bulb at the lower end. The graduation marks are in terms of even per cents and 0.2 fractions, 10 g. of butter being used. The graduated tube is joined to an Erlenmeyer flask by a rubber stopper and is enclosed in a condenser jacket.

Reagent. *Amyl Ester Reagent.* Mix 5 parts of amyl acetate with 1 part of amyl valerianate, both free from water-soluble impurities.

Process. *Distillation.* Introduce into the flask 10 g. (5 or 15 g. for extreme percentages) of the sample weighed on a 13-cm. square of parchment paper. Add 6 ml. of *amyl ester reagent*, connect with the jacketed graduated tube, fill the condenser to the mark, and remove glass stopper *F*. Boil the mixture in the flask, taking care that the steam does not rise higher than the 15% mark before condensing. If the mixture foams, disconnect, cool, add 2 ml. of the *amyl ester reagent*, and proceed with the boiling. Any of the mixture that is carried over into the safety bulb after the water has been driven off causes no injury. When the water is completely removed from the flask as indicated by a crackling noise and a brown discoloration of the liquid, which usually requires 5 to 8 minutes, disconnect the flask and insert the stopper firmly in the tube.

Separation of Water. Repeat a number of times to secure a sharp dividing line and complete separation. Holding the bulb *C* so that the mouth of the test tube within it is directed upward, pour the water out of the jacket and then remove the jacket itself. Invert the tube, thus allowing the water and amyl ester reagent to flow into the graduated portion. Bring about a final separation of the two liquids and collection of all traces of water in the graduated portion by holding the bulb of *C* in the palm of the hand so that the tube is horizontal, with the stoppered end away from the body, then rapidly swing at arm's length. Finally, with the tube inserted (stoppered end pointing downward), rinse down the water adhering to the sides by agitating the amyl ester reagent.

Reading. Take upper and lower readings while slightly warm, observing the same precautions as in reading the fat column in Babcock bottles.

Doran Centrifugal Method.[192] Process. Warm the sample to 38°, stir thoroughly, remove about 10 ml. to a graduated sedimentation tube, and whirl in a centrifuge for a few seconds, taking care that the sample is sufficiently liquid to insure a good reading. Read the amount on the graduation, add about 5 ml. of *gasolene*, and mix thoroughly by inverting two or three times, closing the tube with the thumb, which is not removed until the mixture has drained a few seconds. Whirl again for 15 to 20 seconds and read the amount of non-fatty material at the line of demarcation between the two layers.

Calculation. The percentage of non-fats is obtained by multiplying this reading by 100 and dividing by the volume of butter as first read on the tube. The percentage of butter fat is obtained by difference. Duplicates are said to agree within 0.2% of each other and within 0.5% of gravimetric determinations.

Note. Obviously this method depends on a compensation of errors, as volumes of melted butter and non-fatty matter are not strictly comparable on the weight basis.

Lactic Acid

Hillig Ferric Chloride Colorimetric Method.[193] Reagents. As for the Hillig Method under Milk above.

Process. *Acid Extraction.* Weigh 20 g. of the sample into a 100-ml. beaker, add 15 ml. of water and 0.5 ml. of *1.0 N sulfuric acid*, and heat on a steam bath for 10 minutes with frequent stirring. Neutralize with *1.0 N sodium hydroxide solution* and transfer to an 8-ounce (250-ml.) centrifuge bottle with 15 ml. of water.

Removal of Fat. Add 50 ml. of *washed ether* and mix well, but avoid vigorous agitation. Add 50 ml. of *naphtha*, mix well, centrifuge, and run off the solvent layer as completely as possible through a siphon with the lower end sharply recurved. Repeat the extraction, using 25 ml. each of the *two solvents*. Transfer the residue in the bottle to a 100-ml. volumetric flask with water, add 3 ml. of *1.0 N sulfuric acid*, and heat in a water bath cautiously to expel the ether.

Removal of Proteins. Cool, add *phosphotungstic acid* dropwise with stirring until the proteins are precipitated, then make up to the mark, shake, and filter.

Treat 50 ml. of the filtrate as directed for the Hillig Method under Milk, beginning with the side-head *Ether Extraction*.

Examples: 0.0016 to 0.0557 g. of lactic acid per 100 g. of butter.

Salt

Ogg, Johns, Hoecker, and Hammer Micro Volumetric Method.[194] The method was developed at the Iowa State College for studying the distribution of salt in butter, not for determining the salt content of an entire sample. The procedure is illustrative of delicate technique applicable in principle to solving other problems.

Apparatus. *Simple Binocular Microscope*, magnifying 6 diameters.

Micro Balance.

Micro Spoons, made from platinum foil 7 mm. in diameter fused to platinum wire.

Micro Burets, of 1-mm. bore, filled and discharged by a piece of rubber tubing provided with a plug and screw-clamp, attached to the upper end. A celluloid rule serves as a scale.

Reagent. *Dichlorofluorescein Indicator.* Dissolve 0.1 g. of the dye in 60 ml. of ethanol, make up to 100 ml. with water, then dilute 1 ml. of this solution with 9 ml. of ethanol.

Process. *Charge.* Temper the butter and hold at 13°. Expose a new surface by breaking for each portion removed. Pick off about 0.2 mg. with a needle and place in the tared micro spoon, then weigh spoon and butter together. By using a relatively warm micro spoon, condensation of moisture on a damp day is obviated.

Incineration. Hang the micro spoon on a Nichrome wire drawn across the top of a 15-ml. porcelain crucible, then melt the butter with a microburner, causing it to spread as a thin film. Char the butter, then reduce to ash by heating just to redness.

Titration. Transfer the spoon to a depression in a spot-plate in which has been placed a drop of water, cover with another drop of water, then after several minutes wash with 3 or 4 drops of water, followed by 3 or 4 drops of *ethanol*, restricting the volume to 0.5 ml. Add *standard 0.01 N sodium chloride solution* measuring 10 to 12 cm. (not milliliters) on the scale and 1 small drop of *dichlorofluorescein indicator*, then titrate back with *standard 0.01 N silver nitrate solution* in a dark room under fluorescent light.

Calculation. Deduct for the added sodium chloride and calculate the percentage of sodium chloride in the sample.

Diacetyl and Acetoin

The aroma of butter, tobacco smoke, roasted coffee, chocolate, beer, and honey is

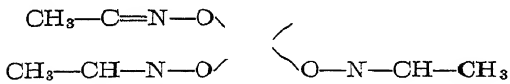
attributed in part to diacetyl (dimethylglyoxal) $CH_3 \cdot CO \cdot CO \cdot CH_3$. *Diacetyl* is formed in ripened cream by the oxidizing (dehydrogenating) action of bacteria on *acetoin* (methylacetyl-carbinol or dimethyl ketone), $CH_3 \cdot CO \cdot CHOH \cdot CH_3$, which may be reformed by the partial reduction (hydrogenation) of diacetyl with zinc and acid.

Schmalfuss Nickel Diacetyldioxime Gravimetric Method.[105] The *diacetyl* obtained by the distillation of a brine extract is heated with hydroxylamine hydrochloride, the reaction being as follows:

$CH_3 \cdot CO \cdot CO \cdot CH_3$ (Diacetyl) $2NH_2OH \cdot HCl$ (Hydroxylamine hydrochloride)

$CH_3 \cdot C(NOH)$ CH_3 (Diacetyldioxime) $\cdot$ $2HCl$

The diacetyldioxime (dimethylglyoxime) reacts with nickel chloride or sulfate to form nickel diacetyldioxime (nickel dimethylglyoxime).



NICKEL DIACETYLDIOXIME

Acetoin, converted into diacetyl by oxidation with ferric chloride, also is determined in the same manner.

APPARATUS. *Distillation Assembly.* Large with 5-liter flask, small with 500-ml. flask; both with provision for a stream of carbon dioxide.

REAGENTS. *Nickel Sulfate Solution*, 1.25%, calculated to $NiSO_4$.

Ferric Chloride Solution, 50%. Dissolve 50 g. of sublimed $FeCl_3$ in water and make up to 100 ml.

A. Diacetyl. PROCESS. *Distillation.* Place 1 kilo of the butter sample, together with 650 ml. of *saturated sodium chloride solution*, in the 5-liter flask of the large distilling assembly, and distil in a current of *carbon dioxide gas*, heating at first at 110°, later at 150°, in an oil bath, until 200 ml. have passed over. Saturate the distillate with *dry sodium chloride* and distil in the small assembly until 8 ml. are collected.

Nickel Diacetyldioxime Precipitation. Prepare in a 150-ml. Erlenmeyer flask a mixture of 50 ml. of water, 2 ml. of *20% hydroxylamine hydrochloride solution*, and 3 ml. of *10% nickel sulfate solution* (calculated to $NiSO_4$). Cool to 0°, add to the cooled mixture the 8 ml. of distillate, mix, and cool for 1 minute. Then add dropwise *1 + 1 ammonium hydroxide* at 0° to just alkaline reaction, close with a rubber stopper covered with a strip of filter paper, mix by rotating, and place in a bath of cold water. Raise to boiling and hold at that temperature for 1.5 hours, then cool for 1 hour in ice water.

Filtration and Weighing. Collect the precipitated nickel diacetyldioxime on a small porcelain Gooch crucible with holes 6 μ in diameter and wash with about 100 ml. of water at 0°. Dry at 110 to 120° to constant weight (about 1 hour), cool for 2 hours in a desiccator over phosphorus pentoxide, and weigh on a micro balance.

CALCULATION OF DIACETYL. Multiply the weight of the precipitate by the factor $\frac{2 \times 86.05}{288.83} = 0.595$, which gives the gammas per gram of diacetyl.

B. Acetoin. PROCESS. Distil in a stream of *carbon dioxide gas* the residue from the second distillation above and test the distillate to insure complete removal of diacetyl. Combine the residue with the residue from the first distillation in the large flask and add through the carbon dioxide inlet tube 50 ml. of *50% ferric chloride solution*. Distil the diacetyl thus formed and proceed as in the determination of diacetyl.

CALCULATION. Multiply the weight of the precipitate by the factor $\frac{2 \times 88.06}{288.83} = 0.610$, which gives the gammas per gram of acetoin.

Schmalfuss and Werner Simplified Modification.[196] For the detection of added diacetyl and acetoin in oleomargarine, as well as their determination in butter, Schmalfuss and Werner use a semi-micro simplified modification of the foregoing method.

A. Diacetyl. PROCESS. Distil 50 g. of the sample in a 150-ml. Erlenmeyer flask with 10 ml. of *saturated sodium chloride solution* and collect 0.55 ml. of distillate in a test tube containing 0.1 ml. of *20% ammonium hydroxide*, 0.05 ml. of *22.50% hydroxylamine hydrochloride solution*, and 0.05 ml. of *1.25% nickel sulfate*, adding more *ammonium hydroxide* if necessary to maintain an alkaline reaction to *litmus*. Redistil the distillate and collect 0.55 ml. of distillate. Cool for 30 seconds and note the intensity of the red color.

Blank. Proceed as in the actual analysis, except that the 50 g. of the sample are omitted. The distillate should be colorless or faintly violet in color.

CALCULATION. Determine the smallest weight of diacetyl that gives the reaction, which in various oils and oleomargarine made with milk was about 0.24 γ/g. and in slightly rancid and highly rancid oleomargarine was 0.32 and 0.48 γ/g. respectively, then judge the amount in the sample accordingly.

EXAMPLES. Milk and skim milk less than 0.24, German butter made in August 0.30, sour cream 0.24, and oleomargarine 1.04 γ/g.

B. Acetoin. Treat in like manner 50 g. of the sample, replacing the sodium chloride solution by 10 ml. of *50% ferric chloride solution*. A red color indicates acetoin.

DIACETYL

Pien, Baisse, and Martin Diaminobenzidine Colorimetric Method.[197] The method depends on the formation of diphenylquinoxaline, from the reaction between diaminobenzidine and diacetyl, which, because of its yellow color, can be compared with standard potassium bichromate solution. It was devised for use in the detection of the addition of diacetyl to butter.

APPARATUS. *Special Distillation Apparatus.*

REAGENT. *Standard Potassium Bichromate Solution.* Standardize against 10 ml. of a solution of 10 mg. of diacetyl in 100 ml. of water treated with the same amounts of reagents as given below.

PROCESS. *Distillation.* Distil 100 g. of butter in the special apparatus devised by the authors or as described in the foregoing method, all the diacetyl being concentrated in 10 ml. of distillate.

Color Formation. Add 0.5 ml. of freshly prepared *2.5% aqueous diaminobenzidine solution* to the 10 ml. of distillate and shake, then add 0.5 ml. of *hydrochloric acid* and shake again; finally allow to stand for 2 minutes.

Color Comparison. Compare with the *standard potassium bichromate solution.*

EXAMPLES. Of 130 samples of butter, 11% contained 0.5 to 1.0, 47% 0.1 to 0.5, and 42% less than 0.1 γ/g. of diacetyl.

Wilson Nickel Dimethylglyoxime Gravimetric Method.[198] REAGENT. *Nickel Sulfate Solution.* Dissolve 15 g. of $NiSO_4 \cdot 6H_2O$ in water and dilute to 100 ml.

PROCESS. *Distillation.* Steam-distil a measured quantity of the sample diluted with 100 ml. of water. Collect about 100 ml. of the distillate in a beaker flask containing 2 ml. of *2 + 8 hydroxylamine hydrochloride solution*, 5 ml. of *2 + 8 sodium acetate solution*, and 20 ml. of water, taking care that the delivery tube dips below the surface of the mixture. Disconnect the condenser and rinse the inside with two or three 10-ml. portions of water.

Nickel Precipitation. Add to the distillate 2 ml. of *15% nickel sulfate solution* and evaporate to dryness on the steam bath. Add 10 ml. of water, warm until the soluble salts dissolve, and let stand 6 to 24 hours. Collect

the precipitate of nickel dimethylglyoxime in a tared Gooch crucible, wash with water, then with 20 ml. of ethanol, and dry to constant weight at 100 or 110°.

CALCULATION. Weight of the precipitate × 0.596 = weight of diacetyl.

PASTEURIZATION

Kay and Graham Test and Scharer Modification. See Milk, Pasteurization (G1) above.

NOTE. Shadwick and Parker [199] report the following results on flashing sour cream by the Kay and Graham method with 24-hour incubation: 85° negative, 82.5° doubtful, 79.5° (and below) positive. When flashed 2.5 to 8 hours at 82.5°, the result was negative. By the Scharer modification, the results were 85° negative, 82.5° negative, 79.5° doubtful, and below 79.5° positive.

VITAMIN A AND CAROTENE

See also Part I, C10, and Part II, G1, above.

Gillam Spectrographic Method.[200] Vitamin A and carotene of butter are both determined on the unsaponifiable matter.

APPARATUS. A *Photographic Instrument*, such as the Hilger E3 quartz spectrograph with photometer; or a *visual instrument*, such as the Hilger-Nutting spectrophotometer.

Gillam found the visual method the better as there is less danger of destruction of the carotenoids by the ultra-violet rays of the light source.

REAGENT. *Antimony Trichloride Reagent.* See Carr and Price Method (Part I, C10).

PROCESS. *Saponification.* Saponify a weighed amount of butter (100 g. of winter or 25 to 50 g. of summer butter; or half of these quantities if the antimony trichloride test is not to be used) of known water content for 30 minutes with a slight excess of colorless *20% alcoholic potassium hydroxide solution* and pour the soaps into about three volumes of water.

Extraction and Solution. Extract the solution with *ether* until no more color is removed. Wash the combined extracts carefully with several portions of water and remove the ether by evaporation in a current of *nitrogen.* Dissolve the residue in *chloroform,* make up to 10 to 20 ml. in a graduated flask, and examine at once.

If the spectroscopic analysis is not carried out immediately, the evaporation of the ether should be completed in a brown glass ampoule and the residue sealed off in nitrogen.

Determination. A. Carotene. Using a solution representing a known weight of butter per 100 ml., measure the intensity of absorption at 455 to 460 mμ either photographically or visually with the apparatus designated above. The value so obtained is expressed as $E^{1\%}_{1\,\text{cm.}}$ (butter) at 455 to 460 mμ and the absorption due to carotene is taken as 94% of this value.

Calculation. To calculate the percentage of carotene in the butter, use is made of the fact that $E^{1\%}_{1\,\text{cm.}}$ at 463 mμ ($CHCl_3$) = 1900 for pure carotene.[201] The results are conveniently expressed as milligrams of carotene per 100 g. of dry matter.

B. Vitamin A. The band shown at 328 mμ by certain fish liver oils is a definite property of vitamin A; it is also exhibited by butter. The absorption at 328 mμ of the unsaponifiable matter of butter is the sum of vitamin A plus carotene and xanthophyl. To obtain the absorption due to carotene plus xanthophyl at 328 mμ, divide the observed value of $E^{1\%}_{1\,\text{cm.}}$ for these substances at 445 to 460 mμ by the factor 6.5. This correction is usually of the order 10 to 25% of the gross absorption at 328 mμ. From value $E^{1\%}_{1\,\text{cm.}}$ (butter) at 328 mμ, due to vitamin A, it is possible to determine the vitamin A content in milligrams per 100 g. of dry butter by making use of value $E^{1\%}_{1\,\text{cm.}}$ = 1600,[202] given by

Carr and Jewell for their richest concentrate. This may need correction if the vitamin A is concentrated further, according to Karrer and Morf [203] who prepared a concentrate of $E^{1\%}_{1\,\text{cm.}}$ at 328 mμ = 1700.

Antimony Trichloride-Spectroscopic Test. Although the results are always low and therefore not recommended for pure butter, Gillam considers the test of value where unsaponifiable artificial coloring matter interferes with the ultra-violet absorption method. When the solution of the unsaponifiable matter of butter is treated with *antimony trichloride in chloroform,* the blue color formed is largely vitamin A, but some small part is due to carotene and a lesser amount to xanthophyl. If, however, the intensity of absorption of the blue solution is determined at 585 mμ with the visual spectrometer, as followed by Heilbron, Gillam, and Morton [204] for liver oils and by Gillam, Heilbron, Morton, Bishop, and Drummond [205] for butter, the value so obtained can be regarded as vitamin A and carotene only, the xanthophyl being negligible.

Calculation. When the absolute carotene value from the direct absorption and the blue value for pure carotene ($E^{1\%}_{1\,\text{cm.}}$ at 585 mμ = 420) are both known, it is possible to calculate that due to carotene. The difference gives the blue value due to vitamin A in terms of $E^{1\%}_{1\,\text{cm.}}$ butter at 585 mμ. Since, according to Heilbron, Heslop, Morton, Webster, Rea, and Drummond,[206] the purest distillates have a value of $E^{1\%}_{1\,\text{cm.}}$ at 585 mμ = 2600 in chloroformic antimony trichloride, the vitamin A content can be calculated in milligrams per 100 g. of butter.

Neal, Haurand, and Luckmann Spectrographic Method.[207] Recognizing the limited application of the biological method, the chemists of The Best Foods, Inc., propose the following technique for the determination of total vitamin A (carotene plus vitamin) potency.

Apparatus. *Hilger Intermediate Quartz Spectrograph,* with *Spekker Photometer,* equipped with tungsten steel electrodes as a light source and accessories as follows: (1) *Quartz Absorption Cells,* Hilger Type C, 1 cm.; (2) *Quartz Kjeldahl-Shaped Flasks,* 25 ml.; (3) *Ultra-Violet Mercury Lamp,* rectifier type quartz arc, supplied by the Cooper Hewitt Electric Co., Hoboken, N. J.

Data Sheet, S–201, compiled by the General Electric Vapor Lamp Co., Hoboken, N. J.

Reagents. *Ether* and *Ethanol,* both redistilled over KOH, the latter with aluminum grains.

Process. *Saponification.* Boil 25 g. of the fat, separated from the sample by melting at 60° and filtering, with 30 ml. of *20% ethanolic potassium hydroxide solution* for 5 minutes.

Ether Extraction. Dilute to about 4 volumes with water, cool in an ice bath, and extract with 180-, 150-, 100-, and 50-ml. portions of *ether* with vigorous shaking, then wash the combined extracts with 6 portions of 150 ml. each of water, merely pouring the first two through the ether. Filter through paper and evaporate to 25 to 50 ml. at first by distillation, then remove the last traces of ether by heating on the water bath in a stream of *carbon dioxide.*

Cyclohexane Solution. Cool the residue to 21°, dissolve in the *solvent,* and make up to exactly 50 ml., then filter, and store in the dark at 4 to 10° not longer than 2 days.

Irradiation. Place half of the cyclohexane solution in a 25-ml. Kjeldahl-shaped quartz flask, stopper with a cork covered with aluminum foil and support the flask on aluminum foil with the cork against the rim of the lamp and the bulb 10 cm. away. Gently tap the flask every 15 minutes. Allow to heat to the full capacity of the lamp short of boiling. Irradiate (1 to 1.5 hours) until both vitamin A and carotene have been destroyed as shown by the Carr and Price test.

A. Irradiated Portion (Spectrophotometric Exposure). Cool to about 21°, filter

the clear colorless irradiated solution, and make the exposure (1-cm. cells) for carotene and that for vitamin A in the non-irradiated other half of the cyclohexane solution, using the irradiated one as control. Expose the plates (Eastman 33) at densities of 0 to 1.50 in increments of 0.05 with exposure up to about 2 seconds and develop with D 72 diluted 1 to 2.

Readings. Read match-points from the same plate, carotene at 4600 Å (M) and vitamin A at 3280 Å (M_1).

CALCULATION. The following formulas for calculation of U.S.P. vitamin A units per pound of butter fat due to carotene (C) and vitamin A (A) respectively are condensed from those given in the paper:

$$C = \frac{M \times 0.94 \times 4.54 \times 1000}{0.50 \times 2.1 \times 0.6}$$

$$A = \quad [illegible] - [illegible]) \times \frac{2140 \times 4.54}{0.50}$$

The figures in the formula are based on the following data: (1) 94% of light is absorption at 4600 Å due to carotene; (2) $E^{1\%}_{1\,\text{cm.}}$ for carotene in cyclohexane is 2100 at 4600 Å; (3) 0.6 γ of β-carotene is equivalent to 1 I.U. of vitamin A; (4) the absorption at 328 $\mu\mu$ due to carotene plus xanthophyl is obtained by dividing the observed value $E^{1\%}_{1\,\text{cm.}}$ for these substances at 455 to 460 $\mu\mu$ by the factor 6.5; and (5) the instrument factor for vitamin A is 2140.

B. Non-Irradiated Portion. Use pure cyclohexane instead of the irradiated solution as control. Compare the match-points at 3280 Å with that obtained in the original determination. The difference between the match-points, using pure cyclohexane and the irradiated unsaponifiable solution as control, should be between 10 and 25%; if greater than 25%, incomplete destruction of the vitamin by irradiation, oxidation, or evaporation of solvent is indicated.

VITAMIN A

See also Part I, C10, and Part II, G1, above.

Fraps and Kemmerer Method for "Spectro Vitamin A." [208] In butter fat these authors (Texas Agricultural Experiment Station) determine (1) total color in terms of carotene (colorimetric method), (2) crude carotene (A.O.A.C. Method), (3) pure carotene (selective adsorption magnesium carbonate method), (4) non-carotene color (total color less pure carotene), (5) artificial color soluble in ether, and (6) spectro vitamin A as follows:

APPARATUS. *Spectrograph,* Bausch & Lomb medium quartz, equipped with a photometer density scale and a silver electrode.

PROCESS. *Saponification.* Reflux 5 g. of butter for 30 minutes with 50 ml. of *12% aldehyde-free ethanolic potassium hydroxide solution* in a stream of *nitrogen.*

Ether Extraction. Take up with 50 ml. of water, cool to 4°, and shake in a 1-liter separatory funnel with 50 ml. of *purified ether* and 150 ml. of cold water. Draw off the ether layer and shake the aqueous layer three more times with 15-ml. portions of *ether.* Wash the combined ether solutions with cold water until free from alkali, dry over *anhydrous sodium sulfate,* and distil off the ether in *nitrogen* under reduced pressure.

Methanol Extraction. Take up the residue in hot *absolute synthetic methanol,* make up to 10 ml. in a volumetric flask at room temperature, cool for 2 hours at −8° in an ice-salt bath, and filter the methanol solution from the crystallized impurities.

Spectrographic Examination. Photograph the absorption spectra of the methanol solution in a 2-cm. tube at a density between 0.6 to 1.1 at 328 mμ, using in the comparison tube a solution prepared in a blank procedure. If the density was too low, repeat with more butter and larger quantities of the reagents. Correct for carotene (approximately

0.03 γ/g.) determined by comparison with 0.1% dichromate solution.

Calculation. Proceed as directed by Bauman and Steenbock,[209] using the Beer-Lambert formula $E = 1/cdD$. For 5 g. of butter, 10 ml. of solution, and a 2-cm. cell, multiply the absorption density by 6.25 to obtain the gammas per gram of vitamin A.

Correction for Artificial Color. When the non-carotene color is over 3 γ/g., correction is necessary. If the artificial color is assumed to be that supplied by nine American dealers and examined by Fraps and Kemmerer, the correction equivalent to 1 γ/g. of carotene will be a density of $2 \times 0.22 \times 0.52 = 0.228$ in a 2-cm. cell compared with 0.03 for carotene. After making the correction, derived as given above, for carotene, deduct from the spectro vitamin A 0.6 γ/g. for each 1γ/g. of artificial color.

International Units. Obtain the international units per gram of vitamin A (I.U.) by the formula

$$\text{I.U.} = (S - 0.5)4 + 1.7C$$

in which S and C are respectively gammas per gram of spectro vitamin A and carotene read from the total color.

In commercial butter fat, use the pure or crude carotene in gammas per gram in place of the total color.

Carotene

See also Part I, C10, and Part II, G1, above.

Barnett [210] determines the carotene in the purified fat of natural-colored butter by diluting with *refined cocoanut oil* to a carotene content between 0.25 and 0.75 γ/g. and reading the color value in a spectrophotometer. The values ranged from 7.3 to 13.0 γ/g.

The values obtained by the Palmer colorimeter method may be converted into carotene values by the factor 0.28.

Tocopherol

See also Part I, C10.

Emmerie and Engel Ferric Chloride-Dipyridyl Colorimetric Method. See Part I, C10.

Emmerie Modification.[211] Proceed as directed in the original method with removal of carotene.

Iodine

See also Part I, C8*b*.

Aitken Alkali-Fusion Method.[212] By this method, contributed from the University of Otago, New Zealand, minute quantities of iodine in butter and other fats may be determined.

Process. *Saponification.* Saponify in a shallow nickel basin 25 g. of the sample by heating for 1 hour in a water bath with 20 ml. of *50% sodium hydroxide solution*, followed by 80 ml. of *ethanol*.

First Ignition. Evaporate on the sand bath in a shallow round-bottom iron dish of such a depth as to allow an air space between the bottom and the nickel basin, avoiding a glow on the bottom. After 3 hours, when the evolution of gases has ceased, heat the melt further for at least 30 minutes until a gray layer of potassium carbonate remains, then cool and wash down the layer with a little water.

Second Ignition. Evaporate to dryness and again ignite for 30 minutes. Take up the residue in about 150 ml. of water, filter hot with gentle suction on a 10-cm. Büchner funnel, and wash. Transfer the nearly colorless filtrate and washings to a clean basin and evaporate to dryness.

Third Ignition. Ignite as before for 15 minutes. When cool, add 8 ml. of *half-saturated potassium carbonate solution*, rub to a paste with the aid of a few milliliters of *90% ethanol*, then add 25 ml. of *95% ethanol*, and stir well into the paste. Decant into a 100-ml. nickel crucible and repeat the extraction

two or three times, using a total of 75 ml. of ethanol. Gently evaporate the ethanolic extract, with the addition of a little water, to dryness, rinse down the salts with a few milliliters of water, and decant the solution into a 25-ml. nickel crucible, repeating two or three times with a total of about 18 ml. of water.

Fourth Ignition. Add 10 drops of *saturated potassium carbonate solution*, evaporate to dryness on the sand bath, place in a larger crucible over bits of pipe clay, and ignite for about 5 minutes in the non-luminous flame.

Ethanol Extraction. Form the residue and a few drops of *saturated potassium carbonate solution* into a paste and extract with successive portions of 0.5 ml. of *ethanol*, decanting into a titration tube (with a platinum wire sealed in the bottom to insure regular boiling) together with a bit of *pumice*, and evaporate to dryness on the sand bath.

Bromine Treatment. To the tube, add 1 ml. of water, acidify with 3 drops of *1% sulfuric acid*, add 3 drops of *freshly prepared saturated bromine water*, evaporate to about 0.3 ml., and cool, then add a minute crystal of *potassium iodide*. If more than 0.4 γ of iodine is present, a yellow color will be evident.

Titration. Deliver *0.01 N thiosulfate solution* from a micrometer screw buret,[213] add a small drop of *freshly prepared starch solution* when the solution is nearly colorless, and finish the titration in diffused light.

EXAMPLE. A sample of butter fat was found to contain 1.09 γ/kg. of iodine; on adding 1.10 γ/kg. of iodine, 2.16 γ/kg. were found.

8. BUTTER SUBSTITUTES

PHYSICAL AND CHEMICAL VALUES OF BUTTER SUBSTITUTES

See also Part II, B2.

The methods for the determination of the physical and chemical values (constants) of butter are the same as for the examination of other animal fats and vegetable oils. They serve chiefly for distinguishing butter from butter substitutes for which there is now little demand except in federal or state regulatory laboratories.

Nature of Substitutes. Oleomargarine is the legal term in the United States for all butter substitutes, animal or vegetable or mixtures of both. In England and some other Europen countries the equivalent term is margarin.

As first prepared by Mége Mouries in France in 1872 and for some time thereafter in other countries, the fatty portion of oleomargarine consisted of oleo oil made from beef suet rendered at a low temperature to avoid a cooked taste, slowly cooled so as to crystallize the higher-melting-point glycerides of the saturated acids, and pressing. The hard cake (stearin) obtained as the by-product, when mixed with cottonseed oil, was suited for a lard substitute of intermediate consistency.

This early product was readily distinguished from butter fat by an almost complete lack of volatile (soluble) fatty acids as determined by the Reichert-Meissl method, although an admixture containing as high as 20% with butter might escape detection because of the wide range for pure butter. When cocoanut oil came into use in vegetable butter substitutes, the differentiation was less sharp and 25 or, in extreme cases, nearly 50% might escape detection.

The following table shows the range in values of the principal fats and oils that enter into the composition of butter substitutes. By the removal of the stearin from tallow, lard, and cottonseed oil, the range of most of the values is extended and by hydrogenation oils are changed into solid fats with radically different values.

In interpreting the results of analysis, it should be remembered that goat's butter fat has a high Polenske number.

	Butter Fat	Oleo Oil	Cocoa-nut Oil	Palm Kernel Oil	Lard	Cotton-seed Oil
Specific gravity, 100°/15.5°						
Minimum	0.865	0.860	0.863	0.859	0.859	0.872
Maximum	0.870	0.863	0.874	0.873	0.864	0.873
Refraction, 40°						
Minimum	40.5	47	33	36	48.6	61.8
Maximum	46.0	50	36	39	51.2	65.8
Saponification No.						
Minimum	220	198	246	242	193	191
Maximum	241	202	268	255	203	195
Iodine No.						
Minimum	26	40	8	13	46	104
Maximum	38	50	9.5	18	70	117
Reichert-Meissl No.						
Minimum	24	0.3	6	4	0.2	0.7
Maximum	34	0.4	8.5	7.6	0.8	0.9
Polenske No.						
Minimum	1.5	0.5	15.5	6.6	0.4	....
Maximum	3.5	0.6	20.5	12	0.6	....
Kirschner No.						
Minimum	20	....	1.6	1.1	....	....
Maximum	28	....	1.9		....	....
Baryta Nos.						
Insoluble (*b*)						
Minimum	242.6	254.5	296.5	303.7	253.5	255.2
Maximum	254.8	257	299.2		259.2	256.9
Soluble (*c*)						
Minimum	50.6	8.7	54.1	32.9	6.3	6.6
Maximum	76.7	9.6	57.6		12.2	10.6
Difference (*d*)						
Minimum	−24.8	+45.5	+38.9	+70.8	+41.5	+44.6
Maximum	−0.7	+47.4	+45.1		+52.9	+50.3
Shrewsbury and Knapp No.						
Minimum	23	19.5	163	....	15.7	....
Maximum	33			....		....
Ethyl Ester No.						
Second distillate						
Minimum	7.1	1.7	41.5	23.1	2.7	....
Maximum	13.4	3.0	43.5		3.2	....
First distillate						
Minimum	21.8	1.0	10.6	1.5	0.6	....
Maximum	28.1	2.7	15.6		1.8	....

Physical Tests

Normal Butter vs. Renovated or Imitation Butter. In addition to the chemical and physical methods for determining the values on the fats separated from the water, ash (including salt), and curd, certain semi-scientific tests performed on the original sample furnish more or less reliable information as to the nature of the product. Several of these were described as household tests in *Farmers' Bulletin 131*, issued by the U. S. Department of Agriculture.

Foam or Spoon Test. Heat cautiously about 3 g. of the sample on a large spoon or casserole over a Bunsen burner or alcohol lamp. Normal butter boils quietly with the formation of abundant foam; renovated butter and oleomargarine sputter and bump like other hot greases. The test is a reliable indication of whether or not the sample contains fat that has previously been melted.

Melting Test. Heat 10 to 20 g. of the sample in a test tube at 50° until melted. The fat of normal butter rises as a clear liquid, whereas that of renovated butter or oleomargarine is more or less cloudy because of curd particles.

Microscopic Test. Press a small portion of the unmelted sample on a microscopic slide with a cover-glass and examine under the compound microscope with moderate magnification. Normal butter shows a more regular distribution of curd particles and greater transparency than renovated butter or oleomargarine in which opaque curd masses are conspicuous. With crossed nicols of the polarization apparatus, normal butter is uniformly illuminated with no evidence of crystalline structure and no play of colors when the selenite plate is interposed, whereas renovated butter or oleomargarine gives evidence of crystalline structure and display of colors with the selenite plate.

The tests are valueless if the sample has been at any time melted.

Waterhouse Clotting Test.[214] Unlike the foregoing tests which distinguish normal butter from renovated or imitation butter, this test distinguishes with some degree of certainty normal and imitation butter from oleomargarine, at least of the type on the American market forty years ago. Information on the deportment of modern oleomargarine containing more or less cocoanut oil or hydrogenated oils is lacking.

Process. Heat nearly to boiling 50 ml. of milk, add 5 to 10 g. of the sample and stir with a wooden stick while the fat is melting. Cool in ice water, continuing the stirring. As the fat of oleomargarine solidifies it usually may be collected with the stick as a clot; the fat of both normal and renovated butter remains granular.

In a modification proposed by Patrick,[215] skim milk is substituted for whole milk and the mixture is heated to the boiling point after addition of the sample.

Reichert-Meissl Number

See also Part II, B2.

Influence of Added Glycerides and Preservatives on the Reichert-Meissl Number. The addition of triacetin and tributyrin to imitation butters has been proposed to defeat the purpose of the Reichert-Meissl method. Of these, according to Fincke,[216] only triacetin answers the purpose, 5% being sufficient to give a Reichert-Meissl number corresponding to that of genuine butter. As a preliminary test, he determines the Reichert-Meissl number, then adds 110 ml. of water to the residue in the distillation flask and repeats the distillation. If triacetin was added, the ratio of the titration numbers of the first and second distillates is raised.

To secure absolute proof Fincke proceeds as follows. Reflux for 1 hour on an asbestos plate 30 g. of the sample with 150 ml. each of water and *ethanol* and a few bits of *pumice stone*, cool, separate the fat from the dilute

ethanol (containing the triacetin), and dry on a bath. Determine the Reichert-Meissl and saponification numbers of the fat thus treated and compare with the same numbers before treatment.

The presence of benzoic and salicylic acid also raises the Reichert-Meissl number. Bemelmans[217] found that neutral lard containing 2% of benzoic acid gave a number of 1.92. Grimaldi,[218] by the use of 4% of this acid, was able to raise the Reichert-Meissl number of a mixture of fats containing only 78% of butter so as to conform to that of pure butter. The same author states that such admixture can be detected by the increase in acidity.

', Polenske, and Numbers

Dyer, Taylor, and Hamence Semi-Micro Modification of the Combined Reichert, Polenske, and Kirschner Methods.[219] The senior author is the well-known research worker of the Rothamsted Experiment Station and a member of the publication committee of the *Analyst*.

Apparatus. The *Assembly* is shown in Fig. 173.

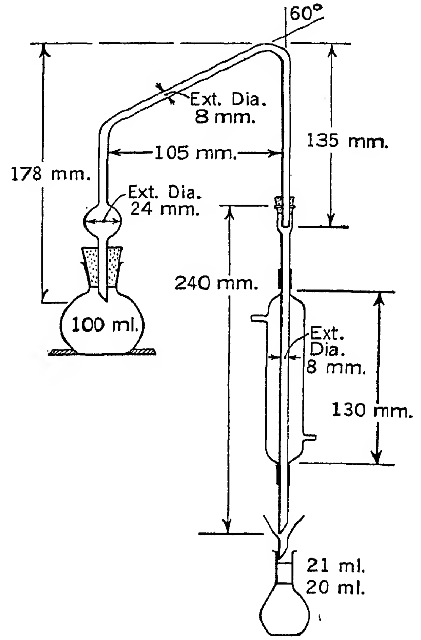
Reagents. *Sulfuric Acid*, 2.5% by volume. Dilute 25 ml. of H_2SO_4 to 1 liter and adjust until 40 ml. exactly neutralize 2 ml. of 50% NaOH solution.

Phenolphthalein Solution. Dissolve 0.5 g. of the indicator in 100 ml. of ethanol and carefully neutralize.

Ethanol. Boil, cool, and neutralize ethanol immediately before use.

Process. *Saponification*. Weigh 1 g. of the butter fat into the 100-ml. distilling flask, add 0.5 ml. of *50% sodium hydroxide solution* and 3.5 ml. of *glycerol*. Saponify by heating over a small naked flame with continual agitation until the liquid becomes clear. Cool somewhat and add cautiously 19 ml. of boiled distilled water and rotate until the solution is complete.

Distillation. Add to the soap solution 10 ml. of *2.5% sulfuric acid* and 0.05 g. of *powdered pumice*. Connect with the still head and heat gently until the insoluble acids have melted completely, then increase



Courtesy of Analyst 1941, **66**, *356*

Fig. 173. Dyer, Taylor, and Hamence Volatile Acids Distillation Assembly.

the heat so as to distil in 5 to 7 minutes sufficient liquid to fill exactly to the mark of a 21-ml. flask.

A. Reichert Number. Cool the distillate in water kept at 15° for 10 minutes and transfer any solid fatty acids on the filter to the flask by gently tapping the neck of the flask, then mix the contents by inverting several times. Filter into a 20-ml. volumetric flask through a 4.5-cm. No. 4 What-

man paper, adjust to the mark, and titrate with *standard 0.02 N barium hydroxide solution*, using *phenolphthalein indicator*.

Calculate the Reichert number by multiplying the number of milliliters of the standard solution used in the titration by 1.05.

B. Polenske Number. Wash the condenser twice with 3-ml. portions of cold boiled water, passing each separately through the beaker, the funnel tube, the 21-ml. flask, and the filter paper that separated the Reichert portion. Dissolve the washed insoluble acids in 3 portions of 3 ml. each of *ethanol*, passing each portion through the filter, funnel, condenser, beaker, and the paper into the 21-ml. flask. Titrate the combined filtrates with *standard 0.02 N barium hydroxide solution*.

The Polenske number is the number of milliliters of standard barium hydroxide solution used in the titration.

C. Kirschner Number. To the neutralized or titrated solution from the Reichert determination, add 0.1 g. of *silver sulfate* and shake at intervals in a dark place for 1 hour. Filter into a dry beaker, place 20 ml. of the filtrate in the 100-ml. flask, add 7 ml. of water, 2 ml. of *2.5% sulfuric acid*, and 2 small pieces of *aluminum wire*. Connect with the distilling assembly and distil sufficient liquid to fill to the mark of a 21-ml. volumetric flask. Cork the flask, mix by inverting several times, then filter through a 4.5-cm. No. 4 Whatman paper into a 20-ml. volumetric flask. Titrate the 20 ml. of distillate with *standard 0.02 N barium hydroxide solution*.

Calculate the Kirschner number (K) by the following formula:

$$K = \frac{k \times 1.1 \times (20 + r)}{20}$$

in which k and r are the number of milliliters of standard solution used respectively for the Kirschner and the Reichert titrations.

Kirschner Number

See also Part II, B2.

Flanders and Truitt Simplified Modification of the Kirschner Method.[220] By omitting the second distillation, substituting silver nitrate for silver sulfate, and operating directly on the fatty acids precipitated as silver salts, the usual process has been simplified.

Reagents. *Chloroform.* Wash if not neutral and filter to remove the water.

Standard Sodium Ethylate Solution, 0.1 *N*. Prepare from cleaned sodium and absolute ethanol. Standardize against 0.1 *N* HCl.

Glycerol-Sodium Hydroxide Solution. See Part II, B2, Reichert-Meissl Number.

Process. *Saponification.* Saponify the fat with *glycerol-sodium hydroxide solution*, distil 110 ml. of the glycerol-soda liquid, and titrate, following the usual procedure for the determination of Reichert-Meissl number.

Silver Precipitation. To the neutralized distillate, add 5 ml. of *10% silver nitrate solution*, stopper the flask, let stand 30 minutes with occasional rotation, and filter on a 9-cm. Whatman No. 41 paper. Allow to drain but do not wash. Transfer the precipitate to a separatory funnel with cold water, rinse the flask with a minimum of water, then with 15 ml. of *0.1 N sulfuric acid*, and pour the acid over the filter into the separatory funnel. Discard the filter after rinsing with a little water.

Chloroform Extraction and Titration. Stopper the separatory funnel, shake vigorously until the precipitate is dissolved, rinse the flask with 15 ml. of *chloroform*, and add to the separatory funnel. Shake thoroughly, let stand until the chloroform clears, and draw off the chloroform layer carefully through a Whatman No. 4 dry paper into a 150-ml. Erlenmeyer flask. Repeat the extraction twice with 10-ml. portions of *chloroform*, allowing a few minutes before drawing off, rinse the filter with 25 ml. additional of

chloroform, then add 3 to 4 drops of *ethanolic phenolphthalein solution* and titrate directly in the chloroform solution with *0.1 N sodium ethylate solution* to a strong end-point.

Cocoanut and Palm Kernel Oils

At the time when the fatty matter of oleomargarine consisted solely of oleo oil mixed perhaps with neutral lard and cottonseed oil, its distinction from butter and its detection when present in butter in considerable amount was readily accomplished by the determination of the refraction and the Reichert-Meissl number, but since cocoanut and palm kernel oils have come into use, especially in European oleomargarine factories, the problem becomes more complicated. These oils are about as much lower than butter in their refraction as oleo oil is higher, therefore a mixture of the two in equal proportions cannot be distinguished from butter by this value. Mixtures of equal parts of oleo oil and palm kernel oil would also have about the same iodine number and saponification number as butter fat. The Reichert-Meissl number of cocoanut oil and of palm kernel oil is about one-quarter that of butter, hence by selecting butter with a high Reichert-Meissl number the admixture of 25% of these oils would readily escape detection by this constant.

To meet these difficulties, methods have been based on: (1) the presence of greater amounts of volatile, but insoluble, fatty acids in cocoanut and palm kernel oils than in butter (Polenske method), (2) the absence of butyric acid in the oils (Kirschner method), (3) the difference in the solubility in water of the barium soaps of the fatty acids (Avé-Lallement method), (4) the difference in solubility of the fatty acids in 50% ethanol (Shrewsbury-Knapp method), and (5) the difference in volatility of the ethyl esters (Hanuš method). In addition certain well-known constants, such as specific gravity, refraction, saponification number, and iodine number, often throw light on the nature of mixtures.

Cocoanut Oil

Hinks Microscopic Method.[221] Process. Dissolve 5 ml. of the clear melted fat in a test tube in 10 ml. of *ether*, pack in ice, and after 30 minutes filter rapidly the ether solution from the solid glycerides through a pleated paper. Evaporate the filtrate to dryness on a water bath, heat the residue on a water bath in a test tube with 3 to 4 volumes of *ethanol* to boiling or until the fat has dissolved, then cool to room temperature and finally at 5° for 15 minutes. Filter the ethanol solution from the separated glycerides and cool to 0°.

The crystals thus deposited from butter, as seen under the microscope (250 diameters), are in round granular masses, whereas from cocoanut oil they are in fine needles. Butter containing as little as 5% of cocoanut oil shows fine, almost feathery crystals attached to the granular masses. The addition of beef, cottonseed oil, and sesame oil, or feeding cocoanut cake, does not interfere, but the presence of lard alters somewhat the form of the butter granules.

Shrewsbury and Knapp 50% Ethanol Volumetric Method.[222] Glycerides of lauric and myristic acids constitute 86% of cocoanut oil. The method devised by Shrewsbury and Knapp is based on the solubility of these acids in 50% ethanol.

Reagent. *Glycerol-Sodium Hydroxide Solution*, 2.0 *N*. Mix 100 ml. of 10 *N* KOH with 500 ml. of glycerol.

Process. *Saponification.* Heat 5 g. of the fat with 20 ml. of *2.0 N glycerol-sodium hydroxide solution*, as in the Reichert-Meissl number, dilute with boiling water, and wash into a separatory funnel with exactly 200 ml. of water.

Fatty Acids Liberation. Add 5 ml. of *1 + 4 sulfuric acid*, shake 1 minute, allow to stand

5 minutes, and draw off the aqueous solution from the insoluble fatty acids.

Separation of Insoluble Fatty Acids. Dissolve the latter in 50 ml. of *90% ethanol*, pour into a flask, and heat to boiling with a bit of *pumice*. Pour the boiling ethanolic solution into the separatory funnel, to which previously had been added 36 ml. of water, then return the mixture to the flask and rinse back into the separatory funnel. Shake for 30 seconds and allow to stand 3 minutes.

Titration. Draw off the ethanolic solution, containing the cocoanut oil acids (chiefly myristic), from the separated acids and titrate 70 ml. with *standard 0.1 N sodium hydroxide solution.* The number of milliliters required is the Shrewsbury and Knapp number.

EXAMPLES. As reported by the authors, butter required 28 ml., oleomargarine free from cocoanut oil 20 ml., and cocoanut oil 163 ml. of standard 0.1 *N* alkali.

9. CHEESE

Composition. L. L. Van Slyke gives the composition of 7-weeks-old cheddar cheese as follows: water 36.06, protein 24.45, fat 34.43, lactose, lactic acid, etc., 0.61, and ash (including salt) 3.61%.

The **U. S. Standards** specify that the name "cheese" unqualified is understood to mean "Cheddar cheese, American cheese, American Cheddar cheese." In addition they specify the following limits for fat: cheddar, pineapple, Limburger, brick, and neufchâtel 50%, gouda 45%, cream 65%. Not more than 39% of water is allowed in cheddar cheese. Of cheeses classed as whole milk or partly skimmed milk, at least 45% of fat is required in Emmenthaler (Swiss) and Camembert.

SAMPLE.

Cut from spherical or drum-shaped cheeses thin wedge-shaped segments extending from rind to center. Divide brick-shaped cheeses in quarters by cutting lengthwise and crosswise and retain one quarter. If preferred, whatever the shape, remove two central cores with the trier, one from top to bottom, the other from side to side, and mix the two.

Prepare the sample by chopping, kneading, grating, or grinding, according to its physical condition. Avoid loss of moisture or weigh before and after preparation so as to correct the analysis for loss.

MOISTURE

Gravimetric Oven Method. Dry 2 g. of the prepared sample in a flat-bottom dish, 5 cm. in diameter, in a boiling water oven. If the dish has a slip-in cover, cooling in a desiccator is unnecessary. A platinum dish permits the determination of moisture and ash on the same portion. Continue the drying until the weight is sufficiently constant for practical purposes.

In the writers' experience, all varieties continue to lose for 24 hours and some continue to lose after 148 hours. The water content, stage of ripeness, and barometric pressure during drying are among the factors affecting loss. Drying for 6 hours, which can be carried out in a working day with time for weighing the portion before and after drying, although often not sufficient to remove the last per cent of water from some varieties, seems a reasonable conventional procedure.

PROTEIN

Determine the nitrogen by wet combustion (Part I, C1*c*) and calculate the protein, using the factor 6.38.

FAT

Two methods were brought to the attention of agricultural chemists by Babcock[223]

in 1892, one the Short copper sulfate gravimetric method, dehydration being effected by grinding with anhydrous copper sulfate, the other an adaptation of the Babcock centrifugal method. The first combines simplicity with accuracy, preliminary drying being avoided, the second is rapid and as accurate as could be expected of a method that dispenses with the chemical balance. Results by both methods in collaborative work during 2 years yielded remarkably agreeing results.

Short Copper Sulfate Gravimetric Method.[223] *Preparation of Mixture.* Grind in a mortar 5 to 10 g. of the sample with 10 to 20 g. of *anhydrous copper sulfate* until the mixture is reduced to a pulverulent dry powder of a light blue color. Transfer the mixture to the inner tube of a fat extractor on the lower end of which has been placed a layer of *anhydrous copper sulfate* 1 to 2 cm. thick. Cover the mixture with a loose mat of asbestos. Place the inner within the outer tube and connect the latter with a weighed flask. Rinse the mortar and pestle with *anhydrous ether* and pour into the inner tube until the total amount nearly half fills the flask.

Extraction. Extract for 4 hours, evaporate the ether, dry in a boiling water oven, and weigh. A second extraction usually yields less than 0.5% additional fat, even when the total fat is 40 to 50%.

Babcock Sulfuric Acid Centrifugal Method. The test, as first modified by Babcock for cheese nearly fifty years ago, today in the writers' opinion in point of accuracy stands at the head of a long list of rapid tests. The centrifuge, glass ware, and other accessories have been perfected, but the method remains practically unchanged and is here described in Babcock's own words:

> The extraction in every case was made with absolute ether and was continued for fifteen hours. The solution of fat was in every case clear, that from the cheese which had been heated being dark colored.
>
> The tests by the Babcock method were made in the cream tubes designed by Mr. Bartlett, of the Maine Experiment Station, . . . the calibration of all the bottles used being carefully tested. The test bottle was first weighed, after which about 8 grams of cheese were put into it and the bottle weighed again. About 15 cc. of hot water were put into the bottles, which were set upon a water bath until the cheese became softened, so that by shaking the bottle a homogeneous emulsion was formed. The test was then completed in the same manner as with milk, the final reading being multiplied by 18 and the product divided by the weight of cheese taken, in grams, for the per cent of fat. A new cheese or a skim cheese will not readily dissolve in this way, but if a few drops of strong ammonia are put into the bottle after the water is added the cheese will soon become softened so that the test can be made. The sulphuric acid should be added slowly with shaking, a little more being used than is necessary for solution.

In the laboratory the bottle is weighed on a chemical balance or pan scales accurate to 0.1 g. For the use of dairymen and inspectors the special cream balance is suited also for cheese.

Lythgoe uses a few drops of *ammonium hydroxide* to aid in the disintegration of the lumps.

Bondzynski-Ratzlaff Wet Extraction Method.[224] This method as regards the shaking out with fat solvents is a combination of the Schmid-Bondzynski method [225] and the Röse-Gottlieb-Patrick method.

Process. Weigh 3 to 5 g. of the sample into a small flask, add 10 ml. of *hydrochloric acid* and a few pieces of *pumice stone* and heat with a small flame until the proteins are dissolved. Cool somewhat and transfer to a Röhrig tube, rinsing the flask with 10 ml. of *absolute ethanol,* then with 25 ml. of *ether*, and finally with 25 ml. of *naphtha.*

Shake, draw off the upper layer into a small filter, complete the extraction by shaking with 2 successive portions of 15 ml. each

of *ether*, evaporate, dry, and weigh, following the instructions given under the Röse-Gottlieb-Patrick Method under Milk above.

In the A.O.A.C. Official Method, 1 g. of the cheese is first digested with 10 ml. of *4% ammonium hydroxide*, then, after neutralizing, 10 ml. of *hydrochloric acid* are added to the mixture and the digestion is completed. Other details are essentially as above given.

Fat Values

In the examination for foreign fats, cheese fat is not so readily separated as butter fat. The process must be one that has no action of the fat that would interfere with subsequent determination of values. Such a process is the Short Method, as described above, but carried out on a larger scale. Complete extraction is not essential.

Colors

See Part I, C11 and C12.

Benzoic and *p*-Chlorobenzoic Acids

See also Part I, C13.

Schwaibold Hydroxylamine Hydrochloride Color Test.[226] Process. *Trituration.* To 30 g. of the sample, add 250 ml. of water and a few drops of *phenolphthalein indicator*, place on a steam bath, and reduce the mixture to a creamy paste (15 to 30 minutes) by means of a glass rod, adding during the operation 8 to 10 ml. of *15% sodium hydroxide solution* in portions. Cool and transfer to a glass-stoppered 500-ml. cylinder.

Ferricyanide-Zinc Sulfate Treatment. Add 30 ml. of *15% potassium ferricyanide solution*, shake well, add 30 ml. of *30% zinc sulfate solution*, and again mix, then make up to 500 ml. and filter on a pleated paper.

Benzene Extraction. Remove 50 ml. of the filtrate to a separatory funnel, add *sulfuric acid* to slight acid reaction, and shake with 50 ml. of *benzene* for 1 minute. Draw off and discard the aqueous layer and add to the benzene solution *0.1 N potassium hydroxide solution* (0.5 to 1 ml.) dropwise until the rose color persists. After shaking vigorously, draw off the aqueous layer to a test tube and evaporate in a current of air introduced through a glass tube reaching nearly to the surface of the liquid. Repeat the extraction of the benzene solution twice with 1 ml. of water, adding the extract to the previous extract and evaporating as before.

Nitrate-Hydroxylamine Treatment. Add to the residue 1 ml. of *10% potassium nitrate in sulfuric acid*, cool, then add 2 ml. each of water and *2% hydroxylamine hydrochloride solution*, followed by 12 ml. of *15% ammonium hydroxide.* Mix well and heat for 5 minutes at 60°.

The presence of benzoic acid is indicated by a red color similar to that of ferric thiocyanate solution, and of *p*-chlorobenzoic acid by a greenish color (under 3 mg.) or a darker color changing to orange (more than 3 mg.).

Lactose

Most varieties of cheese contain scarcely more than traces of lactose, since the small amount derived from the whey that remains in the curd after pressing is largely destroyed by lactic fermentation. Scandinavian whey cheese and certain American cheese mixtures, however, contain notable amounts evident to the taste.

Soxhlet Copper Reduction Method. Rub up a suitable amount of the cheese with successive portions of warm water, decant into a volumetric flask, and finally wash all that remains into the flask. Make up to the mark, shake well, filter on a dry paper, and proceed with an aliquot as described under Milk above.

Vegetable Gums

The use of vegetable gums in place of gelatin as a thickening agent in cream cheese,

cottage cheese, and sour cream has been investigated by Racicot and Ferguson [227] who note that it also permits incorporation of additional amounts of air. The following method, devised by them at the Massachusetts Department of Public Health, Boston, serves for their detection.

Racicot and Ferguson Trichloracetic Acid-Ethanol Tests.[227] A. Preliminary Test. Mix 10 g. each of the sample and water, add 5 ml. of *20% trichloroacetic acid solution*, shake for 1 minute, and filter. To 1 volume of the clear filtrate, add 2 volumes of *ethanol* and mix. If no distinct precipitate or turbidity appears after 30 minutes, no appreciable amount of vegetable gums is present. Locust bean gum or gum tragacanth cause the formation within 30 minutes of a stringy or flocculent precipitate and gum arabic produces a turbidity that settles out as a fine granular precipitate. Decomposition or fermentation may cause a turbidity that persists even after standing overnight.

B. Confirmatory Test. *Precipitation.* Mix well 100 g. of the sample with 100 ml. of water, add 50 ml. of *20% trichloroacetic acid*, shake for 1 minute, and filter. To the entire filtrate add 2 volumes of *ethanol*, mix well, and let stand overnight in a cylinder. Decant the supernatant liquid as completely as possible, leaving not more than 50 ml. in the cylinder. Transfer the remainder of the liquid together with the precipitate to a 50-ml. centrifuge tube and whirl for 5 to 10 minutes or until the supernatant liquid may be poured off without disturbing the precipitate. Wash the precipitate 6 times with 50 ml. of *75% ethanol*, proceeding as above and removing all traces of lactose.

C. Direct Tests. Add to the entire precipitate 15 ml. of water and mix. If the precipitate dissolves, apply the U.S.P. tests for gum arabic, then the following (Part I, C4*a*): add to 8 drops of the solution 5 ml. of *Benedict solution* to exclude lactose and to 1 ml. add *potassium hydroxide solution* and a few drops of *cupric sulfate solution* (Biuret test) to exclude protein. Finally apply the Molisch test for carbohydrates to 1 ml. of the mixture to identify the precipitate as a carbohydrate.

D. Tests after Hydrolysis. Boil gently 2 to 3 minutes 10 ml. of the mixture with 10 ml. of *1 + 2 hydrochloric acid*, cool, neutralize roughly to *phenolphthalein* with *sodium hydroxide solution*, using a *50% solution* at first, then adjust exactly with *0.1 N alkali* or *hydrochloric acid*. Cool and shake with 1 or 2 g. of *decolorizing carbon*, then filter.

Apply the Benedict test to 8 drops of the filtrate, let stand overnight if there is no immediate reaction, and compare with the result by the same test before hydrolysis. A negative Biuret test for protein, a positive Molisch carbohydrate test, and a negative Benedict test before hydrolysis, supplemented by a positive Benedict test after hydrolysis, furnish proof of the presence of vegetable gums.

E. Osazone Test. To 10 ml. of the neutralized solution, add 1 g. of *phenylhydrazine hydrochloride* and 1.5 g. of *sodium acetate*, mix well, and heat in a boiling water bath for 2 hours. Cool and note if osazones are present. Make a microscopic examination, comparing if necessary with the osazones of known purity. Glucosazones are chracteristic of locust bean gum, flat pale yellow crystals resembling maltosazones of gum tragacanth, and small burr-like crystals of gum arabic.

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1. MEAT

Composition. In the following table are given the percentages of lean (muscle), fat, and bone in wholesale cuts of beef as reported by Hall and Emmett:[1]

	Lean	Fat	Bone
Round	65.46	17.12	17.42
Loin	62.96	28.63	8.41
Rib	57.44	26.16	16.40
Flank	39.92	59.94	0.14
Plate	52.21	38.27	9.52
Chuck	71.06	16.41	12.53
Fore shank	51.69	8.25	40.06

The range in composition of muscle free from visible fat is shown in the following table compiled from various sources:[2]

	Water	Muscle Fiber	Connective Tissue	Globulins *	Albumins	Ammo-		Amino Acids	Phosphocarnic Acid	Urea	Fat	Lactic Acid	Carbohydrates ‡	Ash
Min.	72.0	0.6	2.0	12.0	1.5	0.003	0.5	0.8	0.06	0.01	0.5	0.04	0.10	0.8
Max.	78.0	1.3	3.5	16.0	3.5	0.010	1.5	1.5	0.24	0.03	3.5	0.07	0.45	1.2

* Myosin and myogen. † Creatine and creatinine 0.10 to 0.45%; carnosine 0.3 to 0.8%; carnitine 0.05%; methyl guanidine 0.05%; purines 0.10 to 0.25%. ‡ Glycogen 0.0 to 0.2% (horse meat when fresh up to over 1.0%); dextrose 0.10 to 0.25%.

The average composition of beef organs, as given by Powick and Hoagland,[3] follows:

	Water	Protein	Total N	Purine N	Fat	Carbohydrates *	Dextrose	Glycogen	Ash	Phosphorus Total	Phosphorus Organic	Phosphorus Inorganic
	%	%	%	%	%	%	%	%	%	%	%	%
Brains (2)	77.65	10.69	1.71	0.052	7.03	....	0.00	....	1.53	0.345	0.286	0.059
Heart (3)	71.72	17.31	2.77	0.085	9.77	0.36	0.26	0.08	....	0.201	0.140	0.061
Kidney (1)	80.12	15.75	2.52	0.083	2.07	....	0.00	....	1.11	0.230	0.140	0.090
Liver (9)	70.05	20.37	3.26	0.123	2.98	3.53	2.22	1.05	1.32 †	0.358	0.259	0.099
Stomach ‡ (1)	81.08	16.56	2.65	0.023	2.39	....	....	....	0.22	0.040	0.037	0.003
Tongue (2)	.....	18.06	2.89	0.077	....	....	....	....	0.92	0.164	0.073	0.091

* Calculated as dextrose. † Wright and Forsyth: *J. Soc. Chem. Ind.* 1927, **46**, 36T. ‡ Cooked tripe.

SAMPLE

Separate the sample, whether a whole carcass, a meat cut, or an organ, into edible and inedible portions and the former into muscle and adipose tissue, weighing and determining the percentage in each. Grind in a food chopper with a minimum loss of moisture. If the temperature is well below 0° C. or if cold storage is available, freeze the sample until it becomes brittle and grind in the chopper kept sufficiently below the freezing point of the meat juices to prevent thawing due to friction. Keep the sample in cold storage and analyze promptly.

If desired, extract with *naphtha* the fat from a weighed portion of the comminuted sample, dry the residue insoluble in the naphtha, and evaporate the solvent from the extract. Analyze the extracted residue and

the fat separately and calculate the results to the original sample.

Moisture

Air Oven Gravimetric Method. Weigh 10 g. of the sample into a flat-bottom dish, 5 cm. in diameter, preferably of aluminum with a slip-in or slip-over cover, and dry in the air bath at 105° for 2.5 hours. Repeat the heating until the weight is practically constant.

Trowbridge Vacuum Desiccator Gravimetric Method.[4] Add to the inner tube of the Johnson extractor *quartz sand* sufficient to fill two-thirds of the space and a small plug of fat-free cotton. Dry at 103°, keep in a vacuum desiccator a few hours, and weigh in a weighing bottle. Pour the sand into a porcelain dish, add 5 to 10 g. of the sample, mix well, then transfer the mixture to the extractor tube, and wipe the dish clean with the plug of cotton. Place in a vacuum desiccator over sulfuric acid, exhaust to 10 mm. or less, close the stopcock, and allow to stand 24 to 48 hours, with gentle rotation every 3 or 4 hours to mix the diluted top layer of the acid with the strong acid beneath. Repeat the treatment in another desiccator containing fresh acid.. Usually the second period of 24 to 48 hours suffices to secure constant weight.

Indirect Method. Subtract the sum of the protein, fat, and ash content from 100.

Gray Distillation Volumetric Method. See Part II, G7.

The *Gray Apparatus* may be used for determining the moisture content of meat, using, however, 5 g. instead of 10 g. as directed for butter.

Kreis Distillation Volumetric Method.[5] The apparatus and method are similar to those described by Gray during the preceding year. Kreis, however, uses 50 g. of meat or sausage and a *special bulb buret*, with about ten times the capacity of the graduated tube of the Gray apparatus, and *xylol* instead of the amyl mixture.

Protein

Nitrogen Factor Method. If the material is fresh meat or a meat product free from nitrites or nitrates, weigh 3 to 5 g. into a Kjeldahl flask and determine nitrogen by the Kjeldahl-Willfarth or Kjeldahl-Gunning method. In case the sample does not permit of grinding to the proper degree of fineness, use double or triple the prescribed amounts of both sample and acid. After the digestion, transfer to a volumetric flask and use an aliquot for the distillation.

Treat corned meat and all salted meat cured with saltpeter according to the Kjeldahl-Jodlbauer method or, as found necessary by Richardson [6] when a large amount of sodium chloride is present, boil with 10 ml. of *saturated ferrous chloride solution* and 5 ml. of *hydrochloric acid* to eliminate nitrous and nitric nitrogen, then proceed as directed for the Kjeldahl-Willfarth or Kjeldahl-Gunning method.

The reaction with nitric acid liberated from the nitrate by sulfuric acid which Richardson seeks to avoid follows:

$$HNO_3 + 3HCl \longrightarrow 2H_2O + Cl_2 + NOCl$$

Pure Protein

Stutzer Cupric Hydroxide Nitrogen Factor Method. See Part I, C4*a*.

The term *protein* without qualification is used by the present writers for nitrogen times 6.25, which some authors designate *crude protein*.

Water-Soluble Protein

Pennington Centrifugal Nitrogen Factor Method.[7] Although this method was specially designed for chicken meat, it is applicable to the meat of other birds and mammals.

Process. *Extraction.* Weigh 60 g. of finely divided meat into a 500-ml. cylindrical bottle, add 300 ml. of water, and shake

gently for 15 minutes, avoiding an emulsion, then centrifuge for 20 minutes. Decant the supernatant liquid onto a filter and treat again with 300 ml. of water, repeating until the filtrate, measuring 1500 to 2500 ml., does not give a distinct biuret reaction for proteins. Since the process extends through some hours, avoid decomposition by adding a few drops of *thymol solution*. Dark meat requires longer extraction than white, but the time should be limited to 26 hours. Add *0.1 N sodium hydroxide solution* until neutral to *litmus paper* and make up accurately to a definite volume.

Nitrogen Determination. Use 100 ml. or other suitable aliquot, evaporating to 100 ml. before adding the acid.

CALCULATION. Express results as water-soluble protein, calculated by the factor 6.25, or more exactly as water-soluble nitrogen, since nitrogenous constituents other than protein are present.

Emmett Decantation Nitrogen Factor Method.[8] Emmett and his associates have used this method in exhaustive experiments on the composition of mammalian meats.

PROCESS. *Extraction.* Stir 25 g. of the finely ground material, contained in a 150-ml. beaker, with 5 to 10 ml. of ammonia-free water at 15°. Add to the paste 50 ml. of water and continue the stirring for 15 minutes. After allowing to stand 2 or 3 minutes, filter by decantation into a 500-ml. volumetric flask, using a glass rod to press the liquid out of the meat residue. Stir the residue for 5 minutes with 50-ml. portions of water at 15° and decant three times, then with 25-ml. portions three times, allowing to settle 2 to 3 minutes after each. Transfer the residue to the paper and wash three times with 10 ml. of cold water. Dilute to the mark and shake.

Nitrogen Determination. In an aliquot of 25 ml. determine nitrogen and express the result as water-soluble nitrogen or, using the factor 6.25, water-soluble protein.

Other Determinations. Reserve 2 aliquots of 100 ml. each for the precipitation of coagulable proteins, followed in the filtrates by determination of (1) proteoses by the zinc sulfate method, and (2) proteoses and peptones by the tannin salt method and in the filtrate meat bases, also (3) an aliquot of 150 ml. for the determination of creatine and creatinine.

Prepare a new solution for the determination of xanthine bases by the Krüger-Micko Method (below) and check determinations.

COAGULABLE PROTEINS

Grindley and Emmett Nitrogen Factor Method.[9] PROCESS. *Coagulation.* Place a 100-ml. aliquot of the solution, obtained by the Emmett method for water-soluble proteins above, in a 250-ml. beaker and evaporate to about 40 ml. on the steam bath. Add very *dilute acetic acid* until slightly acid to *litmus paper* and boil for a few minutes until the precipitate separates beneath a clear liquid. Collect the coagulated proteins on a filter, wash thoroughly with hot water, taking care that the sides of the beaker are rinsed down with the first portions.

Nitrogen Determination. Remove the filter and coagulum to a Kjeldahl flask, add 25 ml. of *sulphuric acid*, pouring first in small portions into the beaker to dissolve any adhering coagulum, and proceed with the Kjeldahl-Willfarth digestion and distillation in the usual manner.

CALCULATION. Calculate as coagulable proteins, using the factor 6.25, or without calculation express the result as coagulable nitrogen.

NOTE. Trowbridge and Grindley,[10] before boiling, add one-quarter of the amount of *very dilute sodium hydroxide solution* necessary to render the solution neutral to *phenolphthalein.* The tentative method of the A.O.A.C. directs to neutralize, using *phenolphthalein indicator,* then add 1 ml. of *0.1 N*

acetic acid. Otherwise all these methods are practically the same.

Proteoses

Bömer Zinc Sulfate Nitrogen Factor Method.[11] Process. *Precipitation*. In a 100-ml. aliquot of the solution of water-soluble proteins (obtained by the Emmett Method above), precipitate the coagulable proteins, filter, and wash as above. Evaporate the filtrate to a volume of about 50 ml., add 1 ml. of *1 + 4 sulfuric acid* and slowly with stirring *powdered zinc sulfate* to saturation, leaving a small amount undissolved. Filter, wash the precipitate with *saturated zinc sulfate solution*, keeping the funnel covered with a watch

Nitrogen Determination. Transfer the precipitate and paper to a Kjeldahl flask and determine nitrogen.

Calculation. Calculate the proteoses (including albumoses) by the factor 6.25 or report the result as proteose nitrogen.

Note. Moulton[12] found that the higher the percentage of precipitable proteins, the greater the amount of acid required for their precipitation.

Proteoses and Peptones

Schjerning Tannin-Salt Nitrogen Factor Method Modified by Bigelow and Cook.[13] Reagent. *Tannin Solution*. Dissolve 24 g. of highest purity tannic acid in water and make up to 100 ml. Moulton[14] states that often the nitrogen introduced in the tannic acid exceeds that present in the precipitate.

Process. *Tannin Precipitation*. Evaporate a 100-ml. aliquot of the filtrate obtained in the determination of coagulable proteins to about 15 ml. or less, transfer to a 100-ml. volumetric flask, using 50 ml. of *30% sodium chloride solution* to rinse the dish, and shake thoroughly. Cool to 12° in ice water, add 30 ml. of *tannin solution* at 12°, shake, and keep in a refrigerator at about 12° overnight. Adjust to exactly 100 ml., shake, and filter through dry paper.

Nitrogen Determination. Use 50 ml. of the filtrate for the determination. Introduce a correction for the nitrogen in the reagent as determined by a blank.

Calculation. Deduct the corrected weight of nitrogen from the weight of nitrogen determined in an aliquot of 50 ml. of the filtrate from the coagulable proteins. The difference is considered as representing proteoses and peptones, although it consists in part of creatine and probably other impurities.

Express the result either as proteose and peptone nitrogen or as proteoses and peptones, using the factor 6.25.

Notes. Moulton[15] applies the method directly to the extract of meat—not to the filtrate from the coagulable proteins. He calls attention to the defects of the method as applied to meat but considers it is more satisfactory in the analysis of meat extracts.

In the tentative method of the A.O.A.C., the tannin-salt precipitate is assumed to contain gelatin; it should not, however, be assumed that all the gelatin is removed from the meat by cold water.

Collagen and Elastin

(Connective Tissue)

The toughness of meat is due chiefly to the elastin, which with collagen makes up the connective tissue. Collagen on long boiling or autoclaving is converted into gelatin.

Mitchell, Zimmerman, and Hamilton Trypsin Digestion Nitrogen Factor Method.[16] The method, devised at the University of Illinois, depends on the conversion of collagen to gelatin by autoclaving, the removal of muscle proteins by digestion with trypsin, and the determination of the elastin nitrogen in the residue.

Apparatus. *Autoclave*.

Reagent. *Trypsin Solution.* Dissolve 1.5 g. of powdered trypsin and 6 g. of Na_2CO_3 in 2 liters of water.

Process. *Removal of Muscle Tissues.* Free the tissue from all visible fat and surrounding connective tissue and grind in a meat chopper, using the medium cutting plate. Weigh duplicates of 25 to 100 g. each (the smaller amount for high connective tissue content) and macerate for 90 minutes in a small ball mill with 300 ml. of distilled water. Decant onto a 40-mesh sieve, stir the residue in a beaker with 150 to 300 ml. of cold water, and filter through the sieve, repeating six times (total of seven washings).

Conversion of Collagen to Gelatin. Transfer the residue from the sieve to an 800-ml. beaker, dilute to about 400 ml., cover with a watch glass, and autoclave for 2 hours under 16 to 18 pounds pressure. Release the pressure gradually and allow to stand for at least 5 minutes after opening. Decant the hot supernatant liquid through a pleated paper into a 1-liter volumetric flask. Wash the residue back into the beaker with 100 ml. of hot water, boil for a few minutes, and filter. Repeat five times or until the washings give only a constant faint color with the biuret test. Combine the filtrates in a volumetric flask, make to the mark, and determine total nitrogen in an aliquot.

Trypsin Extraction. Wash the residue on the filter back into the beaker with 100 ml. of cold *trypsin solution,* add 3 ml. of *chloroform-toluene mixture,* and digest for 16 hours at 38 to 40°. Heat to boiling, filter through a 120-mesh sieve, and wash the residue three times with hot water, filtering each time through the sieve. Reject the filtrates, transfer the residue to a Kjeldahl flask with water, digest in the usual manner, and determine nitrogen in an aliquot by distillation.

The nitrogen removed by autoclaving and boiling with water is considered to be collagen nitrogen; that in the final residue, elastin nitrogen.

Examples. Results on total nitrogen in per cent of the muscle, and on collagen and elastin nitrogen in per cent of the total nitrogen respectively follow: beef rib 3.19 to 3.65, 2.5 to 9.4, and 4.7 to 8.7; beef shank 3.42, 6.85, and 13.20; pork tenderloin 3.68, 2.65, and 1.75; 2-pound pullet muscle 3.48, 17.9, and 3.9; 3-pound pullet muscle 3.28, 14.75, and 0.80; 4-pound cockerel muscle 3.61, 20.65, and 0.55; 4-pound cockerel thigh muscle 3.69, 12.70, and 2.10; 4-pound cockerel breast 4.14, 6 65, and 1.60.

Collagen

Krylova Autoclave Nitrogen Factor Method.[17] Extract the collagen by autoclaving for 2 hours under 1.5 atmospheres pressure and filtering. After concentration of the filtrate, add *ethanol* followed by *copper sulfate.* Collect the precipitate on a filter and determine nitrogen by the Kjeldahl method.

Gelatin

On long boiling, the collagen of connective tissue is converted into gelatin which consists chiefly of glutin and its derivative glutose.

Long-Boiling Gravimetric Method. Although conventional, this method is of considerable practical value.

Boil with 200 ml. of water for several days the residue from the cold water extraction by one of the foregoing methods, replacing the water as lost by evaporation. When the extraction is practically complete, filter by suction, wash with hot water, and make up the filtrate to 500 ml. Determine nitrogen in an aliquot of 100 ml. and calculate crude gelatin by the factor 5.55.

Evaporate another aliquot to dryness, dry in a water oven, and compare the percentage of residue (hot water extract) with that obtained by calculation from the nitrogen.

Stutzer Short-Boiling Nitrogen Factor Method Modified by Bigelow and Chace.[18] The method was used by Bigelow and Chace [19] in the analysis of preserved meats and by Bigelow and Cook [20] in the analysis of meat extracts.

PROCESS. *Hot Water Extraction.* Boil 10 g. of the sample for 20 minutes with 100 ml. of water, filter, wash with hot water, and evaporate the filtrate to dryness in a dish containing 20 g. of *sand*.

Removal of Ethanol-Soluble Substances. Treat the residue with four 100-ml. portions of *absolute ethanol* and filter after each by decantation with the aid of suction on a Büchner funnel surrounded by ice; then treat the residue with 100-ml. portions of a mixture containing 100 ml. of *ethanol*, 300 g. of *ice*, and 600 ml. of *cold water*, maintaining a temperature below 5°. Continue the treatment until the filtrate is colorless. Remove the asbestos from the funnel and add to the residue in the dish.

Second Hot Water Extraction. Treat the residue, sand, and asbestos with *boiling water* and filter into a Kjeldahl flask.

Nitrogen Determination and Calculation. Determine nitrogen in the filtrate and multiply the percentage of nitrogen by 5.55 to obtain the percentage of gelatin.

Farkas Picric Acid Nitrogen Factor Method.[21] The method was devised at the University of Budapest.

REAGENT. *Brücke Reagent.* Prepare a boiling 10% solution of KI, saturate with freshly precipitated HgI_2, cool, and filter.

PROCESS. *Protein Precipitation.* Heat the solution containing gelatin and soluble protein to 40° and add *saturated picric acid solution*. The proteins are completely precipitated, leaving the gelatin in solution. Filter through paper in a jacketed funnel kept at 40° and wash with warm water.

Gelatin Precipitation. Add to the filtrate 1.5 volumes of *saturated picric acid solution*, cool to 8°, and hold at that temperature 24 hours, thus completely precipitating the gelatin.

Picric Acid Removal. Filter on a pleated paper, wash with *Brücke reagent* until the picric acid is completely removed. In the treatment, mercuric iodide is formed and the gelatin, combined with the hydriodic acid, remains on the paper.

Nitrogen Determination. Remove filter and precipitate to a Kjeldahl flask and determine the nitrogen.

CALCULATION. Express the result in terms of gelatin nitrogen in the total nitrogen or as gelatin in the sample employing a suitable factor.

Than Modification.[22] PROCESS. Grind 0.5 g. of the sample with *sand* and extract the gelatin (collagen) by repeated boiling with water, totaling at least several hours. Combine the extracts and determine the gelatin by the original method.

EXAMPLES. In cuts of beef and veal of first, second, and third quality, the content of gelatin in the total nitrogen was 7 to 10, 12 to 13, and 20 to 21% respectively. In pork it was 10 to 11%.

MEAT BASES AND AMINO ACIDS

Some authors direct to obtain the nitrogen of meat bases plus amino acids by subtracting from the percentage of total nitrogen in the sample the sum of the percentages of insoluble, coagulable, and tannin-salt precipitate (Tentative A.O.A.C. Method), others by subtracting from the percentage of total water-soluble nitrogen the sum of the percentages of nitrogen in the coagulable and tannin-salt precipitate. A simpler course is to calculate the percentage from the weight of nitrogen in the tannin-salt filtrate, remembering, however, that traces of other soluble nitrogenous constituents may be present.

Express results as meat base and amino acid nitrogen.

Amino Acids

Van Slyke Gasometric Method. See Part I, C4*b*, and Part II, H3, below.

Express results as amino acid nitrogen or amino nitrogen.

Glycine

Denigès Alloxan Colorimetric Method.[23] This method, by a distinguished investigator but published in a journal found in only a few libraries, depends on Denigès alloxan reaction.[24] As applied to the dry extract, freed from interfering substances, the details are essentially as follows.

Process. Place less than 0.5 mg. of the extract in a small round-bottom porcelain dish and dissolve in 1 drop of the *alloxan reagent*. Note that a pink color appears after 15 minutes, increasing in intensity up to 2 hours and changing after 4 hours to red-violet. Dissolve the residue in 1 ml. of water, then divide the solution into 2 equal parts. To one half add 1 or 2 drops of *sodium hydroxide solution*, thus forming a violet solution; to the other half add 1 or 2 drops of *5% zinc acetate solution in 2% by volume acetic acid*, thus producing a yellow-orange color.

Compare with standards prepared from pure glycine treated in like manner.

Creatine and Creatinine

The base creatine, $C_4H_9N_3O_2$, and its anhydride creatinine, $C_4H_7N_3O$, occur in muscle, organs, and meat extracts. Both are crystalline substances related to guanidine and arginine. By heating or autoclaving in acid solution creatine is converted into creatinine. In alkaline solution the reverse reaction (hydrolysis) takes place. Creatine is formed in the laboratory by the union of sarcosine (methylglycocol) and cyanamide.

COOH NH$_2$ CO—NH

>CNH

CH

Creatine Creatinine

The creatine content in striated muscle usually ranges from 0.25 to 0.75%, but figures lower than 0.1% have been reported. In organs the content seldom exceeds 0.1%. Ready-formed creatinine occurs in smaller amount than creatine in meat and usually also in meat extract, although in the process of manufacture the former may be formed from the latter.

Folin-Benedict and Myers Picric Acid Colorimetric Method.[25] The Jaffé reaction of creatinine with picric acid,[26] on which this method is based, consists of the formation of reddish brown picramic acid, $C_6H_2(NO_2)_2(NH_2)OH$, by reduction of picric acid, $C_6H_2(NO_2)_3OH$, in alkaline solution, the color being matched against a standard solution of potassium dichromate. The method, as originally proposed by Folin, was specially designed for the determination of creatinine in urine. It has been adapted by Grindley [27] to the analysis of meat and by Bigelow and Cook [28] to the analysis of meat extracts. Several modifications by European authors differ little from the original method.

Apparatus. *Duboscq Colorimeter* or *Nessler Tubes*.

Reagent. *Standard 0.5 N Potassium Dichromate Solution.* Dissolve 24.54 g. of recrystallized $K_2Cr_2O_7$ in water and dilute to 1 liter. An 8-cm. column in the colorimeter or comparison tube corresponds to 9.88 mg. of creatine in 500 ml.

Process. *Coagulable Protein Removal.* Precipitate the coagulable protein in a 150-ml. aliquot of the cold-water extract of 25 g. of meat made up to 500 ml., filter, and wash as described above. Evaporate the filtrate to less than 100 ml. and transfer with rinsing to a 100-ml. volumetric flask. Cool and make

up to the mark. Pipet 50 ml. into a 100-ml. volumetric flask for the determination of total creatinine (creatine plus creatinine) and 25 ml. into a 500-ml. volumetric flask for the determination of ready-formed creatinine.

A. Creatine Plus Creatinine. *Acid Conversion.* Boil down the 50 ml. to about 10 ml., add 10 ml. of *1.0 N hydrochloric acid,* and autoclave for 30 minutes at 117 to 119°. After cooling, add 10 ml. of *1.0 N sodium hydroxide solution,* dilute to the 100-ml. mark, shake, and pipet 5 ml. into a 50-ml. volumetric flask which, when made up to the mark, is equivalent to 50 ml. made up to 500 ml., the standard volume.

Picric Acid Treatment. Add to the solution 3 ml. of *saturated (6 + 500) picric acid solution* and 1 ml. of *10% sodium hydroxide solution,* dilute to the mark, shake, allow to stand 5 minutes, and compare the color with that of *0.5 N potassium dichromate solution* in a Duboscq colorimeter set at 8 mm. or in a Nessler tube.

Calculation. From the reading calculate the total creatinine (creatine plus creatinine) as follows:

$$150 \times 50 \qquad 50 \qquad 25M$$

$$= \frac{4.2128}{M}$$

in which C is the percentage oı total creatinine (creatine + creatinine) and M is the reading in millimeters of the creatinine solution.

If the reading varies more than 3 mm. from that of an 8-mm. column of the standard dichromate solution, measure a new aliquot of more nearly the proper volume, as calculated from the first comparison, and repeat the addition of reagents and the color comparison.

Usually the result on total creatinine is deemed sufficient, but separate determinations of creatine (ready-formed) and creatinine may be made as follows.

B. Creatinine. *Picric Acid Treatment.* To the 25 ml. contained in the 500-ml. volumetric flask, add 30 ml. of *saturated picric acid solution* and 10 ml. of *10% sodium hydroxide solution,* dilute to the mark, shake, and compare with the potassium dichromate solution.

Calculation. Obtain the percentage of creatinine by the formula given above for total creatinine, the double dilution compensating for the omission of the intermediate aliquoting.

If the value for M is outside the limits 5 and 11, measure a new aliquot and compare as above described.

C. Creatine. Subtract the percentage of creatinine from the percentage of total creatinine. Multiply the difference by 1.16 to convert into percentage of creatine.

Calculation. The factor for converting creatine into nitrogen is 0.372.

Ochoa and Valdecasas Micro Modification.[29] Apparatus. *Colorimeter.*

Process. *Acid Conversion.* Weigh a suitable amount of muscle (between 5 and 100 mg.) into a flask, stopper with a cork covered with tinfoil, and heat in an autoclave with 0.2 ml. of *0.2 N hydrochloric acid* at 120° for 24 minutes.

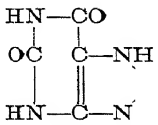
Color Formation. Cool, add 0.2 ml. of *1.2% picric acid,* let stand 5 minutes, and filter. Pipet 0.2 ml. of the clear filtrate into a colorimeter tube and add 0.1 ml. of *5% sodium carbonate solution.*

Color Comparison. After 5 minutes, compare with a solution prepared by mixing 5 ml. of a *standard solution* of 20 mg. of *creatinine* in 1 liter of *0.2 N hydrochloric acid* with 5 ml. of *1.2% picric acid,* followed, when a turbidity appears, by 5 ml. of *5% sodium carbonate solution.*

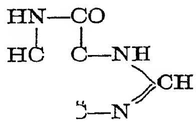
Purines

Constitution. The four purines which have been identified in meat are xanthine, hypoxanthine (oxypurine), adenine, and

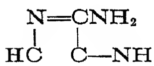
guanine. The basic substance, purine, does not occur in nature.

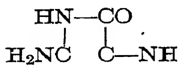


XANTHINE



HYPOXANTHINE

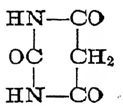




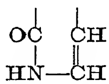
ADENINE

The purines of tea, coffee, and cocoa (theobromine, caffeine, theophylline, and tetramethyluric acid) are considered at the beginning of Part II, I, Alkaloidal Products.

The purine group is allied to the pyrimidine group of which barbituric acid and cytosine are familiar examples.



BARBITURIC ACID



CYTOSINE

Hitchings and Fiske Copper Sulfate Nitrogen Factor Method.[30] The method (Harvard University), designed especially for deproteinized filtrates, serves to prepare a solution of the total purines in a form suitable for individual determinations.

PROCESS. Place the solution containing 3 or 4 mg. of purine nitrogen in a 50-ml. Pyrex conical centrifuge tube, dilute to about 30 ml., and neutralize to *phenolphthalein*. Heat in a boiling water bath, then add 0.8 ml. of *saturated sodium bisulfite solution* and 1 ml. of *10% copper sulfate solution*. Heat 3 minutes, centrifuge, precipitate the purines, and wash twice with 10-ml. portions of hot water. Suspend the precipitate in 3 ml. of *3 N hydrochloric acid*, heat to boiling cautiously over a micro burner flame, and add 15 ml. of hot water. Return the tube to the boiling water bath, and precipitate the copper by passing *hydrogen sulfide gas* through the solution for 3 minutes. Finally cool, rinse into a 25-ml. volumetric flask, dilute to the mark, mix, and filter.

Determine the purines in the solution by suitable methods.

ADENINE AND

Jorpes Copper Bisulfite Nitrogen Factor Method.[31] Jorpes (Karolinska Institute, Stockholm) bases his method for the determination of the two purines in nucleic acid, in the absence of their corresponding oxypurines, on the instability of guanine and the stability of adenine toward permanganate.

PROCESS. *Hydrolysis.* Hydrolyze 2 (4) g. of the sample for 2.5 hours with 90 ml. of *5% sulfuric acid* in a water bath. Cool, dilute to 100 (200) ml., and make alkaline with *ammonia*.

Precipitate the purine bases with *ammoniacal silver solution*, wash the precipitate three times with *dilute ammoniacal silver solution* in a centrifuge tube, treat the precipitate with hot *1.0 N hydrochloric acid*, filter to remove the silver chloride and make up to 100 ml. in a volumetric flask with *1.0 N hydrochloric acid*.

A. TOTAL PURINE NITROGEN. Precipitate the total purine bases in a 7.5- or 10-ml. aliquot of the silver filtrate with *copper bisulfite reagent*. Filter, wash, and determine the nitrogen in the precipitate.

B. ADENINE NITROGEN. Pipet a 15- or 20-ml. aliquot of the silver filtrate into a porcelain dish, add 30 to 50 drops of a *permanganate solution* of such a strength as to oxidize completely the guanine, and heat over a free flame. Filter, wash, and determine adenine nitrogen in the precipitate.

C. GUANINE NITROGEN. Obtain the

guanine nitrogen by subtracting the adenine nitrogen from the total purine nitrogen.

Guanine and Xanthine

Hitchings Phenol Reagent Colorimetric Method.[32] Apparatus. *Colorimeter.*

Reagents. *Phenol Reagent.* See Folin and Ciocalteu Macro Modification of the Folin and Denis Phosphotungstic-Phosphomolybdic Colorimetric Method for Tryptophan (Part I, C4*b*).

Standard Guanine Hydrochloride Solution. Dissolve 29.4 mg. of guanine hydrochloride ($C_5H_5N_5O \cdot HCl \cdot H_2O$) in 100 ml. of 0.1 *N* HCl; 1 ml. = 0.1 mg. of nitrogen (0.001428 *mM* of guanine).

Process. *Extraction.* Grind the tissue in a meat chopper or with sand, extract with 10 volumes of *ice-cold 5% trichloroacetic acid solution,* allow to stand 2 to 4 hours at 5° with occasional stirring, and filter.

Pipet an aliquot of the filtrate into a conical-tipped centrifuge tube, add 0.1 volume of *4 N sulfuric acid,* heat 2 hours in a boiling water bath, add a drop of *phenolphthalein,* and neutralize with *concentrated sodium hydroxide solution.* Make just acid with *dilute sulfuric acid* and return the tube to the boiling water bath.

Free Purine Precipitation by Krüger and Schmid Method.[33] For each gram of tissue represented in the aliquot, add 0.15 ml. of *saturated sodium bisulfite solution* and 0.1 ml. of *10% copper sulfate solution.* Mix and allow the tube to remain in boiling water until the precipitate coagulates and begins to brown (2 to 3 minutes); if it does not turn brown, add additional small quantities of the reagents, avoiding a large excess. After complete precipitation, centrifuge and wash twice on the centrifuge with hot water.

Suspend the precipitate in *3 N hydrochloric acid,* using 0.4 ml. per gram of tissue, and heat to boiling. Add 2 ml. of hot water per gram of tissue, place the tube in hot water, and run a rapid stream of *hydrogen sulfide gas* through the mixture for 3 minutes. Cool, transfer to a volumetric flask, dilute to the mark, mix, and filter. Evaporate an aliquot to dryness on the water bath with the aid of a stream of air, using for the final 0.2 ml. a water bath temperature not over 40°.

Dissolve the purine hydrochlorides in an amount of water equivalent to about 4 ml. per gram of tissue. Precipitate with 0.1 ml. of *10% copper sulfate solution* and 0.08 ml. of *saturated sodium bisulfite solution* per gram of tissue represented. Wash and treat as in the previous precipitation.

Color Formation. Place a quantity of the unknown solution, containing between 0.0007 to 0.0028 *mM* of guanine or xanthine in a test tube with a 25-ml. mark, boil until the water is expelled, and dilute to 1 ml. Pipet into a similar tube 1 ml. of the *standard.* To both tubes add 1.5 ml. of *phenol reagent* and 8 ml. of *saturated sodium carbonate solution,* dilute to the mark, mix, and place in a beaker containing water at 40 to 50°. Allow to cool 20 minutes.

Color Comparison. Compare in a colorimeter.

Calculation. Calculate the guanine, xanthine, or both together (chromogenic purine) on the assumption that there is a uniform distribution between tissue residue and extracting medium and a tissue water content of 80%.

Examples. Chromogenic purine: rabbit liver 0.62, rabbit kidney 0.62 *mM* per kilo. Cat and mouse skeletal muscle 0.08 and 0.05 *mM* respectively. No figures on edible muscle are given.

Adenine and Hypoxanthine

Bruhns Sodium Picrate Nitrogen Factor Method.[34] The method depends on the relative insolubility of adenine picrate and the solubility of the corresponding hypoxanthine salt.

I. Krüger and Schmid Modification. Parnass,[35] in his studies on the formation of ammonia in muscle, employed this modification.

Process. *Acid Extraction.* Grind a suitable amount of the muscle with *quartz sand,* dilute to 25 times the volume of the sample and add sufficient *1% sulfuric acid* to change the color of *Congo red test paper* to blue. Autoclave for 4 hours at 115°, add *sodium hydroxide solution* to faint alkaline reaction, then add *acetic acid* to faint acid reaction and filter while hot.

Hydrogen Sulfide Precipitation. Run *hydrogen sulfide* into the filtrate with heating, filter, and dilute with 25 ml. of water for each 10 g. of the charge.

Ammoniacal Silver Precipitation. Add *ammonium hydroxide* just to alkaline reaction and precipitate the purines with *ammoniacal silver nitrate solution,* avoiding more than a faint ammoniacal reaction. The adenine silver precipitate is gelatinous, whereas the hypoxanthine precipitate is granular. Centrifuge, wash once with *dilute ammonium hydroxide,* mixing thoroughly to distribute the precipitate over the sides of the centrifuge tube, and allow to stand overnight in a vacuum desiccator over dilute sulfuric acid to remove the last traces of ammonia. Take up the precipitate in about 20 ml. of warm water, precipitate the silver with *dilute hydrochloric acid,* centrifuge, wash, and dilute the solution to 50 ml. in a volumetric flask.

Purine Nitrogen Determination. Remove an aliquot of 5 ml., neutralize with *sodium carbonate,* add *magnesium oxide* to alkaline reaction, and heat to remove the ammonia, then determine nitrogen by the Kjeldahl method.

Picrate Precipitation. Concentrate the remainder of the hydrochloric acid solution on the water bath, then remove the remainder of the water first in an air current, then over soda lime in a vacuum desiccator. Dissolve the purine hydrochlorides in hot water, filter, dilute with water to a concentration of 3 mg. of purine to not over 5 ml. of solution, and precipitate with 2 ml. of *saturated sodium picrate solution.* Centrifuge, wash with 1 ml. of water, and combine the solution and washings (fraction B). Dissolve the precipitate in *0.1 N sodium hydroxide* (fraction A).

Separation of Adenine and Hypoxanthine. Transfer fraction A to a glass-stoppered cylinder containing 25 ml. of water and 2 ml. of *10% hydrochloric acid,* add *benzene,* and shake, thus dissolving any precipitate of adenine picrate as it forms. Treat fraction B with water, *hydrochloric acid,* and *benzene* in the same manner. Continue the treatment of both fractions with *benzene* until shaking with *sodium hydroxide* yields no more picric acid.

Fraction A contains the adenine contaminated with guanine; fraction B contains the hypoxanthine contaminated with xanthine.

Dilute both solutions to 45 ml., remove the ammonia from 5 ml. of each by boiling with *magnesia,* and determine nitrogen in the residue by the Kjeldahl method, titrating with *0.01 or 0.02 N acid.*

Calculation. Using the proper factors, calculate the content of adenine and hypoxanthine.

II. Hitchings and Fiske Volumetric Modification.[36] These authors (Harvard University) have greatly simplified the process.

Apparatus. *Logan Assembly* (Fig. 174)[37] or its equivalent for heating in a current of air. Glass wool is placed in the bottom of the heating unit.

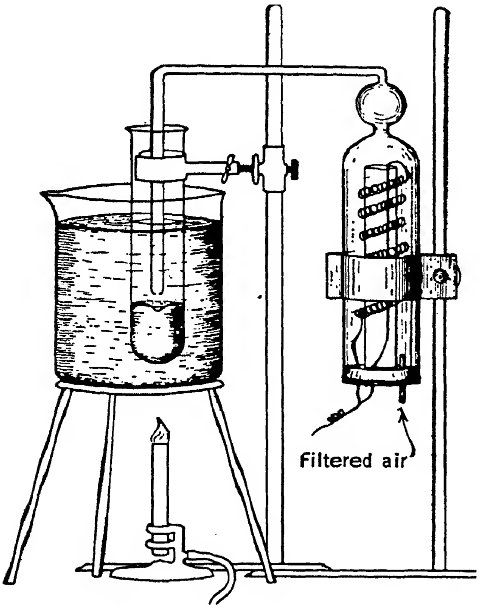
Reagents. *Sodium Picrate Solution.* A saturated solution adjusted by adding picric acid solution so that not more than 0.2 ml. of 0.02 *N* NaOH solution is required in titrating 10 ml. of the solution to an orange color with phenol red. Dilute 50 ml. to 100 ml. for washing.

Standard Sodium Hydroxide Solution, 0.02 *N,* free from carbonate and silicate. Keep in a tightly closed paraffined bottle.

Phenol Red Solution, 0.05%. Adjust if

necessary so that 4 drops of the indicator in 10 ml. of water require no more than 0.01 ml. of 0.02 *N* acid or alkali to produce the full color change.

PROCESS. *Concentration.* Place in a large-lipped Pyrex test tube (200 x 25 mm.) the solution of the sample, containing 1 to 4 mg.



Courtesy of the Author and J. Biol. Chem. 1930, **86**, 762

FIG. 174. Logan Concentration Assembly.

of adenine nitrogen and not more than 5 mg. of adenine plus hypoxanthine nitrogen. If the starting material is purine hydrochloride-hydrochloric acid solution obtained by decomposition of the copper precipitate, remove the excess of hydrochloric acid by heating in a current of air in a suitable apparatus, preferably that devised by Logan. Avoid charring by turning off the heat after the volume is reduced to about 5 ml. Dissolve the residue in 5 ml. of water, adding if necessary 1 to 2 drops of *0.5 N hydrochloric acid* and warming to obtain a clear solution, then cooling to room temperature.

Picrate Precipitation. Add to the solution 1 ml. of adjusted *sodium picrate solution* and filter at once through a small filtration tube with a mat of ashless filter paper pulp.[38] Wash twice with 1-ml. portions of the *diluted picrate solution,* then transfer the filtration tube to the mouth of the original test tube, add 3 ml. of hot water, stir, and finally poke the paper and precipitate into the test tube, rinsing with hot water to a volume of about 8 ml.

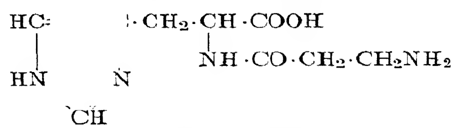
Titration. Add 2 drops of *phenol red solution*, heat to boiling, and titrate at the boiling point with *0.02 N sodium hydroxide solution,* added from a micro buret, to the first distinct red tint.

CALCULATION. Use the equation: 1 ml. of 0.02 *N* sodium hydroxide solution = 1.40 mg. of adenine nitrogen.

NOTE. The quantitative precipitation of guanine requires a higher concentration of sodium picrate and a longer time than that of adenine.

CARNOSINE

Gulewitsch, who with Amiradžibi[39] discovered carnosine, found that the base is β-alanine-histidine. Although neutral to phenolphthalein, Broude[40] showed that it reacts as a monobasic acid when titrated by Sørensen's formol method. It crystallizes as needles and is precipitated by phosphotungstic acid and acid mercuric sulfate.



CARNOSINE (Barger[41])

Gulewitsch Cupric Hydroxide Gravimetric Method.[42] The method as first proposed by Gulewitsch was improved by Mauthner[43] in von Fürth's laboratory and by von Fürth

and Hryntschat,[44] then some years later by Kuen of Vienna University as described herewith. At best it has the disadvantages of all isolation methods.

Kuen Modification.[45] Reagents. *Hopkins Reagent.* Add to 100 g. of $HgSO_4$ slowly with stirring 1 liter of 5% H_2SO_4 and filter from the insoluble basic sulfate that forms.

Wash Liquid. Mix 120 ml. of ethanol, 50 ml. of 10% H_2SO_4, and 30 ml. of ether.

Process. *Extraction.* Grind the fresh sample in a meat chopper after removing visible fat and connective tissue. Extract a weighed portion three times with water at 70° (but not higher). Unite the extracts, add *10% acetic acid* until acid to *litmus paper*, and evaporate to small volume on the water bath. Filter to remove the flocculent precipitate that forms and wash with hot water until the washings do not respond to the diazo reaction.

Mercury and Barium Precipitation. To the clear filtrate add *ethanol* until a slight precipitate forms, then *ether* until a test made on a portion with *Hopkins reagent* yields a precipitate that settles completely, then add to the main solution *Hopkins reagent*, avoiding a large excess in which the precipitate is soluble. After allowing to stand 24 hours, filter, and wash with *wash liquid*, then with *ethanol* and *ether*. When thoroughly dry, remove the precipitate from the filter, grind, and suspend in water.

Barium Precipitation. Add a little *dilute sulfuric acid*, decompose with *hydrogen sulfide*, and add *baryta water* until the solution is weakly alkaline and the precipitates of mercuric sulfide and barium sulfate settle well, then filter. Remove the barium from the filtrate by *carbon dioxide gas*, evaporate to small volume, and filter.

Copper Precipitation. Dilute the clear filtrate with 1 ml. of water for each gram of meat taken and decompose the carnosine with *freshly precipitated cupric hydroxide*, shaking for 20 minutes. If all the cupric hydroxide dissolves, add more, then remove the excess by centrifuging.

Acetone Precipitation. To the deep blue solution of carnosine-copper, add *acetone* until a slight turbidity forms. After 24 hours collect the impure flocculent carnosine-copper on a glass Gooch crucible. If the filtrate has more than a faint blue tint, precipitate again with *acetone*. Dissolve the carnosine copper with *10% ammonium hydroxide* into a crystallizing dish, thus leaving impurities behind on the crucible. Concentrate the solution *in vacuo* or by standing in the air. Collect the crystals of carnosine-copper on a weighed Gooch crucible and dry over calcium chloride to constant weight.

Kuen Histidine Picrolonate Gravimetric Method.[46] Process. Treat as in the Kuen Modification of the Gulewitsch Method, up to the side head *Copper Precipitation*, then proceed as follows.

Acid Hydrolysis. To the concentrated solution, add *hydrochloric acid* and heat on a boiling water bath for 5 hours, then evaporate to full dryness on the water bath *in vacuo*, thus completely removing the acid.

Formation of Histidine Picrolonate. Take up the residue in water, filter, add an *ethanolic solution of picrolonic acid* equivalent to the nitrogen content, and evaporate the ethanol on the water bath, then collect the crystals of histidine picrolonate on a glass Gooch crucible, dry at 100°, and weigh.

Examples. In beef and horse meat, Kuen reports respectively as follows: copper hydroxide gravimetric method 0.31 and 0.26 to 0.38; copper hydroxide colorimetric method 0.40 and 0.42; histidine picrolonate method 0.31 and 0.28 to 0.35; diazo method 0.35 and 0.36%.

Clifford Diazo Colorimetric Method.[47] Although the results by Koessler and Hanke,[48] Hunter,[49] and Kuen[50] do not support conclusions of Miss Clifford (Kings College for Women, Kensington, England), that the carnosine content of the muscle differs with the

species and Kuen finds her results by the diazo method much too high, Kuen's own results by the diazo method (see his modification of the Gulewitsch method above) do not differ greatly from those by the copper and the picrolonate methods.

Apparatus. *Duboscq Colorimeter.*

Reagents. *Diazo Reagent.* See Hanke Colorimetric Method for Tyrosine, Part I, C4*b*.

Color Standard (Koessler and Hanke).[48] Mixture of 1 ml. of 0.5% Congo red solution and 1.1 ml. of 0.1% methyl orange solution added separately to 250 ml. of water, then diluted to 500 ml.

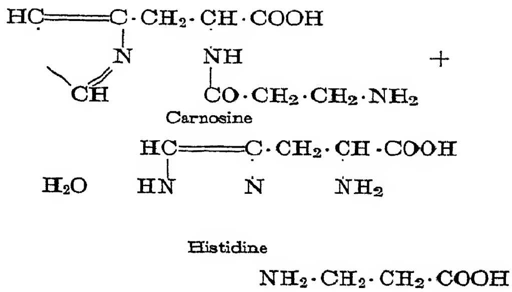
Process. *Protein Extraction and Precipitation.* Treat a weighed amount of the finely comminuted tissues with a known volume of water at 60 to 90° for 15 to 60 minutes, filter on a dry paper, and squeeze out the pulp. Add 2 to 10 ml. (depending on the quantity of material used) of *20% m-phosphoric acid* and allow to stand 2 to 24 hours to precipitate the protein. Filter, neutralize an aliquot in a graduated flask to *litmus* with *10% sodium hydroxide solution*, and make up to a known volume with water.

Color Formation. Place in a dry beaker 10 ml. of *1.1% sodium carbonate solution*, $2 - X$ ml. of water, and from a pipet 4 ml. of *diazo reagent*, and mix by shaking. After 1 minute, add X ml. of the solution of the unknown.

Color Comparison. Place the diazotized solution in one cup of the colorimeter, set at 20 mm., and the *color standard* in the other cup. When the color of the latter has reached its maximum intensity (about 5 minutes after mixing), move the standard until it matches the unknown. Take readings and alter the value of X until, with unknown at 20 mm., the standard matches at 30 mm.

Calculation. When X is equivalent to 1 ml. of 0.01% carnosine solution, the unknown and standard match as above and the amount of extract required to give this color is taken to contain 0.0001 g. of carnosine.

Broude Acrylic Acid Iodometric Method.[51] The β-alanine split off from carnosine by hydrolysis reacts with nitrous acid to form β-lactic acid which in turn is converted into acrylic acid by distillation with sulfuric acid, thus:



$$NH_2 \cdot CH_2 \cdot CH_2 \cdot COOH \text{ less } NH_2 \rightarrow$$

$$\underset{\beta\text{-Lactic acid}}{CH_2OH \cdot CH_2 \cdot COOH} \rightarrow \underset{\text{Acrylic acid}}{CH_2{:}CH \cdot COOH}$$

The last steps are bromination and iodimetric titration. Since the yield is only 86.3%, a correction must be made.

Reagent. *Bromine.* Dissolve 2.5 ml. of bromine and 70 g. of KBr in water and dilute to 1 liter.

Process. *Nitrite Treatment.* Place in a Kjeldahl flask the extract containing 0.1 to 0.14 g. of carnosine and close with a double-bored cork stopper carrying a small separatory funnel and a delivery tube connected with a rubber tube provided with a mercury seal. Add to the solution 0.4 ml. of *glacial acetic acid* free from bromine reacting substances. After expelling the air from the flask by *carbon dioxide* introduced through the separatory funnel, add *2.69% sodium nitrite solution* equivalent to three to four times that required for the reaction, followed by water to 15 ml. total volume.

Reduction. Mix well, allow to stand 24 hours at room temperature, then shake for 5 minutes and add 5 ml. of *49.7% sodium hy-*

droxide solution, rinsing the separatory funnel and the walls of the flask with 15 ml. of water. Add 0.5 g. of *aluminum foil* cut into 1- to 2-mm. squares, allow to stand for 20 hours, during which time the foil dissolves except for a slight brown precipitate of iron hydroxide.

Distillation. Transfer the liquid to a 500-ml. Würtz (round-bottom) flask connected with a spiral condenser, rinsing with 40 ml. of water, add a small spoonful of *talcum powder*, then a mixture of 4 ml. of *sulfuric acid* and 4 ml. of water, followed after a few minutes by 78 ml. more of *acid* to a concentration of about 1 + 1. Incline the flask over a hole in a piece of asbestos board and distil alternately into 50-ml. cylinders with continual replacement of the liquid with water until 500 ml. have been collected, also 100 to 150 ml. additional for a check on the completion of the removal.

Titration. Add to the mixed distillates 20 to 25 ml. of *0.1 N bromine reagent*, followed after 10 to 15 minutes by 3 ml. of *20% potassium iodide solution* and 3 ml. of *25% sulfuric acid*, then titrate the separated iodine with *0.05 N thiosulfate solution*.

CALCULATION. Use the formula: 1 molecule of carnosine ($C_9H_{14}O_3N_4$) = 1 molecule of acrylic acid and 2 atoms of bromine. Correct the result by adding 15.9 (= 13.7 × 100/86.3)%.

AMMONIA

Folin Aeration Method Modified by Pennington and Greenlee.[52] APPARATUS. A train consisting of (1) an *air purification flask* with a safety bulb containing concentrated sulfuric acid, connected by a four-way cock with four series of flasks, each series consisting of (2) a *liter reaction flask*, (3) an *empty flask* to intercept spray, (4) a *250-ml. flask* containing standard 0.1 *N* acid, and (5) a *100-ml. flask* to catch the acid carried over mechanically. The four series are joined by a four-way tube with (6) an *air pump* operated by an electric motor and provided with an anemometer.

PROCESS. Weigh 25 g. of a ground sample into each reaction flask (2), add 1 g. of *sodium carbonate*, 250 ml. of water, and 25 ml. of *ethanol* and run a suitable volume of *standard 0.1 N acid* into the 250-ml. flask (4). Draw 8000 cu. ft. of air through the train during 3 to 6 hours. Titrate back the 0.1 *N* acid and calculate the weight of ammonia liberated and absorbed.

Falk and McGuire Modification of the Folin and Bell Method.[53] Weigh 2 to 4 g. of meat, add 10 ml. of water and 1.25 ml. of *10% sodium hydroxide solution*, and aerate in the Folin apparatus for 2 hours, absorbing the ammonia in *0.01 N sulfuric acid*.

Substitute, if desired, 10 ml. of *20% sodium carbonate solution* for the sodium hydroxide solution, in which case aerate 3 to 4 hours.

EXAMPLES. Fresh meat gave 0.03 to 0.1 mg. of ammonia nitrogen per gram of meat; this increased on decomposition at room temperature during 24 hours or longer to 0.3 to 0.4 mg. per gram.

Richardson and Scherubel Distillation Method.[54] **Process A.** Weigh 100 g. of the sample into a distillation flask, add 10 g. of freshly calcined *magnesia* (MgO) and 450 ml. of water, and distil until 200 ml. have been collected. Replace the water driven out and repeat the distillation ten times, titrating after each distillation with 0.1 *N acid*, using *phenolphthalein indicator*.

After the first distillation, the originators of the method obtained only 0.030 and 0.033% of ammonia from fresh and frozen meat; after 10 distillations the totals were 0.095 and 0.088% respectively.

Process B. Extract 100 g. of the sample three times with 150 ml. of *60% by volume ethanol*. Distil the combined extracts and titrate as in Process A.

The results after the first distillation were about one-third as high as by Process A and

in subsequent distillations the increase was insignificant.

Although results by A and B are not absolute, they were found useful in comparative spoilage experiments where there was a marked increase.

Weber Method.[55] Weigh 20 g. of the well-mixed sample into a 450-ml. Erlenmeyer flask, add 250 ml. of water, and shake for 3 hours in a shaking machine. Add a few drops of *thymol solution* to prevent spoilage and store in the refrigerator overnight. On the following day filter by pouring into a linen bag and manipulating with the hands, repeating the treatment until the biuret test gives a negative reaction, the time required being a whole day and the bulk nearly 2500 ml. Make up to exactly 2500 ml. and filter through a dry 38.5-cm. pleated paper. Use the first 750-ml. portion of the filtrate for the determination of ammonia, the next 600 to 800 ml. for the determination of water-soluble nitrogen, and 200 ml. for the determination of coagulable protein nitrogen.

Nitric Acid

(Nitrates)

Ferrous Chloride Test. Reagent. *Ferrous Chloride Solution, Saturated.* Allow hydrochloric acid to react on an excess of iron nails in a flask provided with a Bunsen valve. Filter when the evolution of gas ceases.

Process. Shake 5 g. of the finely ground sample with 10 ml. of water and filter. To a few milliliters of the filtrate in a test tube, add with shaking and cooling under the tap an equal volume of *sulfuric acid*, then overlay with *saturated ferrous chloride solution.*

The presence of nitrates is indicated by the formation of a dark-colored zone between the two liquids.

Schloesing-Schulze Nitric Oxide Gasometric Method. Boil 50 g. of the sample with successive portions of water, filtering after each until the nitrates are removed. Add *sodium hydroxide solution* to the combined filtrates to slight alkaline reaction, evaporate to about 10 ml., and proceed according to the Schulze Modification of the Schloesing Method (Part I, C4*f*).

Nitrous Acid

(Nitrites)

The presence of nitrites in meat cured with the addition of saltpeter was brought out in the investigation of the bleaching of flour by nitrogen peroxide. Some, if not all, of this passes again into nitrate during aging, extraction, and the Tiemann-Schulze process.

Griess-Ilosway Quantitative Method. Shake with water and determine nitrites as described under Flour, Part II, A2.

Thiocyanates

Brodie and Friedman Nitric Acid-Ferric Nitrate Colorimetric Method.[56] Apparatus. *Colorimeter.*

Reagents. *Ferric Reagent* (Crandall and Anderson).[57] Dissolve 100 g. of $Fe(NO_3)_3 \cdot 9H_2O$ in 1 liter of water, add 50 ml. of HNO_3 with shaking, and dilute to 2 liters.

Standard Thiocyanate Solution. Dissolve about 14 g. of $Na_2S_2O_3 \cdot 5H_2O$ in 1 liter of water. Standardize from time to time against $AgNO_3$ solution and store in a brown bottle at refrigerator temperature. As needed, dilute an aliquot of this stock solution.

Process. *Solution.* Weigh into a 125-ml. Erlenmeyer flask 0.5 to 1.0 g. of finely comminuted wet tissue or 100 to 300 mg. of pulverized dry tissue, cover with 20 ml. of *4% ethanolic potassium hydroxide solution*, and digest under a reflux condenser on the steam bath for 30 minutes. Transfer to a 200-ml. evaporating dish, rinsing first with *ethanol*, then with water, and finally again with *etha-*

nol. Evaporate on the steam bath until the ethanol has been removed, then wash with small amounts of water into a 25-ml. graduated cylinder. Add a drop of *phenolphthalein indicator*, dilute to exactly 20 ml., and pour into a 125-ml. Erlenmeyer flask. Rinse the cylinder with exactly 3 ml. of water and add to the main solution. Neutralize with *4 N nitric acid*, noting the exact number of milliliters required and add exactly 0.5 ml. additional.

Tungstic Acid Precipitation. Add dropwise 1 ml. of *10% sodium tungstate solution* with shaking, then sufficient water to a total of 10 ml. of washings, nitric acid, and tungstate solution. This accurate measurement of washings and reagents is to overcome the difficulty of measurement of the total solution of 30 ml. because of the foaming. Stopper, shake vigorously, allow to stand 10 minutes, filter through a dry paper, and transfer an aliquot of 25 ml. to a 100-ml. beaker, ignoring any turbidity. Neutralize with *10% aqueous potassium hydroxide solution* and add 0.5 ml. in excess.

Norit Clarification. Add to the solution 1 g. of *norit charcoal*, heat to boiling with constant stirring, filter while hot into a 100-ml. beaker, and wash with four 5-ml. portions of water. Repeat the norit treatment if any color remains.

Color Formation. Evaporate the solution to about 5 ml., cool, and add 0.5 ml. of *4 N nitric acid*. Transfer to a 25-ml. glass-stoppered graduated cylinder, make up to 20 ml., then add 4 ml. of the *ferric reagent*, and mix well.

Color Comparison. Transfer the solution to the tube or cup of the colorimeter, filtering if the acidification produced a precipitate. Prepare a color standard in a glass-stoppered cylinder by accurately measuring an amount of the *stock sodium thiocyanate solution*, containing approximately the same amount of thiocyanate as in the unknown, adding 1.5 g. of *potassium nitrate* and 0.5 ml. of *4 N nitric acid*, diluting to 20 ml., and mixing with 4 ml. of the *ferric reagent*. If the volume of the standard differs from that of the unknown, add 1 volume of the *ferric reagent* to 5 volumes of the solution, and *potassium nitrate* to the same concentration as that in the unknown.

REMARK. Although the ferric thiocyanate reaction is extremely sensitive, an unidentified substance, present in the tissues or formed during the treatment, gives a yellow color with the reagent which interferes with the comparison when less than 250 γ of thiocyanate per gram is present in the wet tissues. However, with some experience as little as 75 γ can be satisfactorily determined.

EXAMPLES. Blood 4070 to 4260, muscle 560 to 630, liver 810 to 860, and heart 860 to 1440 γ/g., dry basis.

In the analysis of a sample, whether an entire carcass, a wholesale or retail cut, or a restaurant portion, separate and weigh not only the edible and inedible portions but also the muscle from adipose tissue (and in certain cases from the connective tissue) and determine fat in each.

Use either the portion in which moisture was determined or dry (preferably *in vacuo* or in hydrogen) a new portion of sufficient weight (e.g., 10 g.) to allow for coarseness of the sample. If dried in an open dish in a water oven, avoid long heating since oxidation may introduce a much greater error than the failure to remove the last traces of moisture, if indeed it is moisture that is driven off by protracted heating.

Extract with anhydrous *ether* in the usual manner (Part I, C2*c*).

ACIDS

The chief acid of meat is lactic (α-hydroxypropionic) acid formed by enzymic fission of

carbohydrates and the compounds of carbohydrates with phosphoric acid. Other acids present are succinic, fumaric, oxalic, acetic, and propionic, also ascorbic acid and the higher acids of the fats. It is customary to calculate the acidity in terms of lactic acid.

Direct Titration Method. Boil gently for 5 minutes 50 g. of the finely divided sample with 100 ml. of water, filter on a Büchner funnel, wash with hot water, cool the filtrate, and titrate with *0.1 N alkali* (0.1 ml. = 0.0090 g. of lactic acid), using *phenolphthalein as indicator*.

Calculate as free lactic acid.

Mondschein Oxidation Iodometric Method.[58] According to Mondschein (Vienna University), one-third of the lactic acid is held back by the coagulum in the direct titration method.

Process. *Coagulum Solution.* To the precipitate of coagulated proteins, obtained by the preceding method, add 50 ml. of water and 10 ml. of *10% sodium hydroxide solution*, and boil cautiously, avoiding loss by frothing. When the solution is practically complete, add 100 ml. of *saturated sodium chloride solution*, continue the boiling, and saturate with *solid sodium chloride*. Filter by suction and wash the precipitate with hot *saturated sodium chloride solution*.

Coagulum Reprecipitation. Add to the filtrate *sulfuric acid* to faint acid reaction and boil.

Lactic Acid Oxidation. Add 5 ml. of *sulfuric acid*, make up to 500 ml. in a volumetric flask, and filter through a dry paper. Heat 250 ml. of the filtrate to boiling and add *0.1 N potassium permanganate solution* until all the lactic acid is split up into acetaldehyde, carbon dioxide, and water according to the reaction of von Fürth and Charnass.[59]

Iodometric Titration. Add *standard 0.1 N potassium bisulfite solution* equivalent to the potassium permanganate previously added, mix, and titrate after 15 minutes with *standard 0.1 N iodine solution*.

Calculation. Express results in terms of percentage of combined lactic acid; 1 ml. of the iodine solution = 0.005 g. of lactic acid. Make a blank determination and correct the result.

Lactic Acid

Lehnartz Acetaldehyde Iodometric Method.[60] Quantities as small as 0.02 mg. may be determined by the following procedure based on the Embden [61] and the Hirsch-Kauffmann [62] methods.

Process. *Mercuric Chloride Precipitation.* Powder 0.1 g. of the sample in liquid air, transfer to a centrifuge tube containing 5 ml. of *4% hydrochloric acid*, precipitate the proteins with 2 to 3 ml. of *5% mercuric chloride solution*, allow to stand several hours, then add 17 ml. of water and store overnight in the refrigerator. Centrifuge, decant, remove the mercury from the solution by a stream of *hydrogen sulfide*, and expel the excess of the latter by a current of air.

Copper-Lime Precipitation. Filter into a 35-ml. volumetric flask, add to the filtrate 2 ml. of *10% cupric sulfate solution*, neutralize with *33% sodium hydroxide solution*, and precipitate with 5 ml. of *milk of lime*. Dilute to the mark, shake, transfer to a centrifuge tube, shake repeatedly for 30 minutes, centrifuge for 10 minutes, decant, and filter.

Oxidation to Acetaldehyde. Transfer to a Kjeldahl flask an aliquot equivalent to 90% of the lactic acid taken or, if the content is large, an aliquot representing not more than 0.5 mg. Neutralize with *25% sulfuric acid*, then add an amount sufficient to bring the acid content to 0.5%, followed by 2 ml. of *5% manganese sulfate solution* and a little *talc*. Add *0.002 N potassium permanganate solution*, at a rate of 40 to 50 or 20 to 30 drops per minute (depending on the lactic acid present), to the appearance of a brown color.

Distillation. Attach the flask to a glass-joint condenser, heat on an asbestos plate, and distil the formaldehyde into a receiver

containing 10 ml. of *0.02 or 0.01 N potassium bisulfite solution* cooled in ice respectively for 0.5 or less than 0.2 mg. of lactic acid.

Titration. Add *0.04 or 0.02 N iodine solution, changing near the end-point to 0.005 or 0.002 N* delivered from a micro buret graduated to 0.01 ml. Decompose the aldehyde-bisulfite compound in the titrated solution by a few grams of *sodium bicarbonate* or preferably *disodium monohydrogen phosphate,* shake well, and repeat the titration with *iodine solution.*

CALCULATION. Use the equation: 1 ml. of 0.002 *N* iodine solution = 0.9 mg. of lactic acid.

von Fürth and Charnass Method. The original method and modifications designed for milk analysis are given in Part II, G1.

I. Tanaka and Endo Modification.[63] APPARATUS. *Kumagawa-Suto Extraction Apparatus.*

REAGENTS. Those given in Part II, G1; also $(NH_4)_2SO_4$ and Li_2CO_3.

PROCESS. *Protein and Fat Removal.* Bring to a boil 15 ml. of *30% potassium hydroxide solution,* add to the solution a suitable amount of the sample, and continue the boiling 5 minutes. Cool, neutralize with 30 ml. of *15% sulfuric acid,* and make up to exactly 60 ml. in a cylinder. Precipitate the proteins with 25 g. of *ammonium sulfate* and filter. To exactly 60 ml. of the filtrate, add 10 ml. of *15% sulfuric acid,* 20 ml. of water, and a small amount of *lithium carbonate* and extract for 4 hours with 120 ml. of *ether,* then remove the ether by distillation and drying.

Oxidation and Titration. The oxidation with *sulfuric acid* and *permanganate,* the distillation of the acetaldehyde thus formed into a solution of *sodium bisulfite* in a current of *carbon dioxide,* and the iodometric titration are carried out essentially as in the modification of the von Fürth and Charnass method given in Part II, G1.

II. Ernst and Takács Modification.[64] The procedure embodies features of both the Lehnarz and the Tanaka and Endo modifications.

III. Matakas Modification.[65] A mixture of *5% sodium metaphosphate* and *0.5 N sulfuric acid* is substituted for 5% metaphosphoric acid of earlier authors.

Barker and Summerson *p*-Hydroxydiphenyl Colorimetric Method.[66] When acetaldehyde formed by the action of concentrated sulfuric acid on the lactic acid is treated with *p*-hydroxydiphenyl in the presence of cupric ions a highly specific colored compound is formed. Barker and Summerson of the Medical College of Cornell University have applied these reactions in a method that permits the determination of lactic acid in a solution containing 5 to 10 γ/ml. with an error of less than 0.1 γ/ml.

APPARATUS. *Photoelectric Colorimeter,* with a filter having a peak transmission at about 560 mμ.

PROCESS. *Removal of Proteins.* Remove proteins by any of the common procedures in which trichloroacetic acid, tungstic acid, zinc hydroxide, or cadmium hydroxide is employed.

Copper and Calcium Treatment. To 1 to 5 ml. of the protein-free filtrate, containing 20 to 100 γ of lactic acid, contained in a centrifuge tube, add 1 ml. of *20% cupric sulfate solution* and make up to exactly 10 ml. with water. Add 1 g. of *powdered calcium hydroxide* and shake vigorously. If the bright blue color of cupric hydroxide is not obtained, add more *calcium hydroxide.* Allow to stand at least 30 minutes with occasional shaking, then centrifuge.

Color Formation. Pipet into a test tube (18 to 23 mm. inside diameter) 1 ml. of the clear supernatant liquid from below the surface, taking care to exclude any solid matter from the surface film, and wipe the outside of the pipet clean. Add 0.05 ml. of *4% (crystalline) copper sulfate solution,* then run in from a buret, with mixing but not cooling, exactly 6 ml. of *sulfuric acid.* Place the test tube in a

boiling water bath and after 5 minutes remove to a water bath cooled to below 20°. To the cooled mixture, add from a pipet exactly 0.1 ml. of *alkaline p-hydroxydiphenyl solution* (1.5% in 0.5% NaOH), mix well, and incubate in water at 30° with gentle shaking. After 30 minutes or longer, heat in boiling water for 90 seconds, thus dissolving the excess of reagent to a clear solution, and finally cool in water at room temperature.

Color Measurement. Take the reading in a photoelectric colorimeter, using a filter (Rubicon No. 565 or Klett No. 56) with a peak transmission of about 560 mμ. For the initial setting of the colorimeter, use either *sulfuric acid* alone or the reagent blank.

CALCULATION. The graph (Fig. 175) shows the range in proportion of lactic acid concentration to color intensity as obtained with the Evelyn (2 — log G) and Klett-Summerson (direct reading) photoelectric colorimeters, both curves having been corrected for the reagent blank values.

Winnick Ceric Sulfate Micro Diffusion Method.[67] The method was developed at Wayne University for blood, urine, and tissues.

APPARATUS. *Conway Diffusion Unit.*[68]

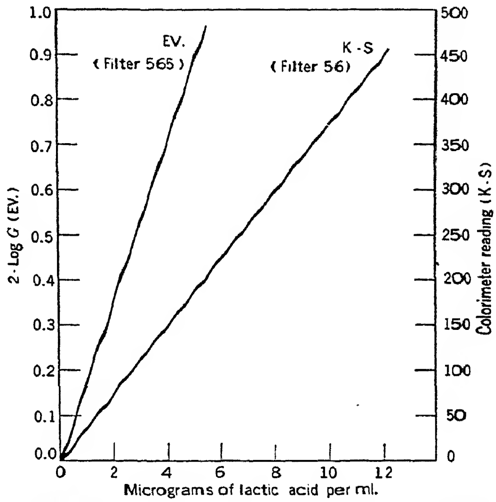
REAGENTS. *Acidified Zinc Sulfate Solution.* Dissolve 18 g. of $ZnSO_4 \cdot 7H_2O$ in 1 liter of 0.166 N H_2SO_4.

Ceric Sulfate Reagent. Saturated solution in 2 N H_2SO_4.

PROCESS. *Extraction.* Freeze the tissue *in situ* with *liquid air* and pulverize in a chilled crusher.[69] Place 0.5 to 0.6 g. of the chilled powder in a stoppered tared flask containing 2 ml. of *acidified zinc sulfate solution*, stopper, shake well, and weigh again. Add 1 ml. each of *10% cupric sulfate solution, 0.333 N sodium hydroxide solution*, and *5% calcium hydroxide suspension*, then centrifuge.

Ceric Sulfate Oxidation and Micro Diffusion. Pipet 3 ml., or less if the lactic acid content is high, of the supernatant liquid into the outer chamber of the diffusion unit. Place in the central chamber 1 to 1.5 ml. of approximately *0.25 M sodium bisulfite solution.* Grease the outer rim of the unit and deliver into the outer chamber from a wide-tipped pipet 1 ml. of *ceric sulfate reagent.* Immediately seal with the glass cover and mix by rotating.

Titration. After 5 hours at room temperature, or 2 hours at 50°, remove the glass cover, add a small drop of *starch solution*, and



Courtesy of the Authors and J. Biol. Chem. 1941, **138**, 541

FIG. 175. Barker and Summerson Lactic Acid Graph.

oxidize the excess of bisulfite with *1 N iodine solution.* As the end-point is neared, transfer small fractions of a drop of the *iodine solution* from the buret tip to the solution with the tip of a stirring rod. When a permanent light purple color is reached, add 0.2 to 0.3 g. of powdered *disodium phosphate* to dissociate the acetaldehyde-bisulfite compound and titrate the liberated bisulfite with *standard 0.003 N iodine solution.*

CALCULATION. Correct the volume of the final extract to include the water content of the tissue, assuming a content of 80%; 1 ml.

of 0.003 *N* iodine solution = 0.135 mg. of lactic acid.

Pyruvic Acid

Krishna Acetaldehyde Volumetric Method.[70] Small amounts of pyruvic acid (2 to 20 mg.) are reduced by *zinc* and *sulfuric acid* in the presence of a trace of *cupric sulfate,* then the resulting lactic acid is oxidized to acetaldehyde which is determined by Clausen's iodometric method.

Case Hydrazone Colorimetric Method.[71] The method depends on the red color formed when the 2,4-dinitrophenylhydrazone of pyruvic acid is dissolved in ethanolic potassium hydroxide, after eliminating the error due to solubility in water by extraction with ethyl acetate and the interference of other hydrazones by treatment with sodium carbonate solution in which they are insoluble.

Apparatus. *Colorimeter.*

Reagent. *Hydrazine Reagent:* 1% 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid.

Process. *Deproteinization.* Treat the tissue or tissue extract with *trichloroacetic acid,* added to make a final concentration of 2 to 5% and filter. If more than 5 mg. of pyruvic acid is present in 20 ml. of the filtrate, dilute, make to a definite volume, and employ an aliquot of 20 ml.

Hydrazine Treatment. To the solution or aliquot, add 5 ml. of *hydrazine reagent* and allow to stand 2 hours at room temperature.

Ethyl Acetate Extraction. Whether or not a visible precipitate has been formed with the hydrazine reagent, shake out the liquid, contained in a glass-stoppered 50-ml. separatory funnel, with 20 ml. of *ethyl acetate,* followed, after separation, by one or more additional 10-ml. portions of the *solvent,* thus completely decolorizing the aqueous layer which is then discarded. Neutralize with *solid calcium carbonate* the united ethyl acetate extracts, containing the hydrazones, the excess of the hydrazine, a certain amount of hydrochloric acid, and trichloroacetic acid. Decant into a glass evaporating dish, wash the calcium carbonate with *ethyl acetate* until colorless, add to the bulk of the liquid, and evaporate to 1 to 2 ml.

Sodium Carbonate Treatment. After removing the dish from the bath, stir with 20 ml. of *toluene* and again transfer to the separatory funnel. Thoroughly shake with 5 ml. of cold *25% sodium carbonate solution,* thus removing the pyruvic acid, which imparts a brown coloration. Repeat the extraction with fresh portions of *sodium carbonate solution* until no more color is removed. Add to the combined sodium carbonate extracts *hydrochloric acid* dropwise to acid reaction, thus precipitating the 2,4-dinitrophenylhydrazone of pyruvic acid as a lemon-yellow suspension. Extract in a separatory funnel with successive 10-ml. portions of *ethyl acetate* until the aqueous layer is colorless (20 ml. are usually sufficient), then evaporate the ethyl acetate solution to dryness in a glass dish.

Color Formation. Cool, add to the yellow residue a drop or two of water, then dissolve in *5% ethanolic potassium hydroxide solution* to a red solution varying in intensity with the pyruvic acid content, and dilute further with the *potassium hydroxide solution* to a suitable volume for color comparison (about 50 ml. for each milligram of pyruvic acid originally present in the sample).

Color Comparison. Use for the comparison a stock solution of *pyruvic acid 2,4-dinitrophenylhydrazone in ethyl acetate,* 1 ml. of which is equivalent to 0.1 mg. of pyruvic acid.

Phosphoglyceric Acid

Rapoport Naphthoresorcinol Colorimetric Method.[72] This procedure, developed at the S. Canning Childs Hospital and Research Institute of Vienna for the analysis of blood, is applicable to muscle, liver, and yeast.

Apparatus. *Pulfrich Photometer.*

Reagent. *Naphthoresorcinol Solution,* 0.1% in H_2SO_4.

Process. *Color Formation.* Introduce a solution containing 50 to 500 γ of phosphoglyceric acid into each of two Wassermann tubes, add to each 0.5 ml. of *hydrochloric acid* and evaporate to dryness on a water bath. To one tube, add 2 ml. of freshly prepared *0.1% naphthoresorcinol solution in sulfuric acid,* and to the other tube add 2 ml. of *sulfuric acid,* and continue the heating for 1 hour. Transfer the solutions to 25-ml. flasks, rinsing the tubes with *sulfuric acid.* Also run a blank determination, using 2 ml. of the reagent.

Color Comparison. Make the comparisons in the 5-mm. cell of the Pulfrich photometer, using filter S 61.

With blood, extracts of organs, and yeast, measure a quantity of a *1 + 5 trichloroacetic acid* filtrate, containing 0.25 to 2.50 mg. of glyceric acid into a small centrifuge tube, neutralize to *methyl orange,* and precipitate with 2.5 ml. of *saturated lead acetate solution* with the addition of 0.5 volume of *ethanol.* Centrifuge, wash the precipitate twice with 3 ml. of water, warm on a water bath, and pass *hydrogen sulfide.* Filter into a 10-ml. flask, dilute to volume, and run determinations on 2-ml. aliquots as before.

Calculation. Obtain the gammas of phosphoglyceric acid (P) by the following formula:

$$P \qquad E \times 100$$

in which E is the extinction coefficient of the unknown and 0.2 is the extinction coefficient of 100 γ of the pure substance.

Sugars

Fresh muscle contains a very small amount of reducing sugar and sugar-cured meats contain sucrose. Direct determination of sugars in a hot water extract of the meat yields low results, owing to the reducing action of creatine and possibly other nitrogenous constituents.

Smith Picric-Phosphotungstic Copper Reduction Gravimetric Method.[73] Smith (Meat Inspection Laboratories, Kansas City, Kan.) eliminates the interfering substances by precipitation with picric acid and phosphotungstic acid and removal of the excess of phosphotungstic acid with hydrochloric acid. His experiments indicate that the presence of a slight excess of hydrochloric acid does not materially vitiate the results.

Process. *Solution.* Remove the fat as completely as possible from the finely divided sample and boil 50 g. of the lean portion in a beaker with 150 ml. of water for 12 to 20 minutes. Cool and remove any solid fat from the top of the liquid.

Precipitation. Decant into a 250-ml. flask, containing 1 to 5 g. of *solid picric acid* and 15 to 20 ml. of *20% phosphotungstic acid solution,* pressing the residue to remove as much of the liquid as possible. Stir the residue with 25 ml. of hot water, cool, and decant, pressing out the liquid as before. Repeat until the flask is filled nearly to the mark, shake, cool, complete the volume, and filter.

Direct Copper Reduction. Pipet 150 ml. of the filtrate and 10 ml. of *8 + 2 hydrochloric acid* into a beaker, mix, filter, and determine immediately the reducing sugars as dextrose in 25 ml. of the filtrate, equivalent to 4.69 g. of the sample.

Invert Copper Reduction. To an aliquot of 50 ml. of the filtrate, add 2 ml. of *hydrochloric acid,* invert, neutralize, and determine the reducing power as dextrose by the Munson and Walker or Allihn Method (Part I, C6*a*).

Calculation. Obtain the sucrose by multiplying by 0.9 the difference in the reducing power before and after inversion.

Hoagland Phosphotungstic Copper Reduction Gravimetric Method.[74] The distinctive feature of this method is precipitation of creatinine and other nitrogenous substances

with phosphotungstic acid alone without addition of picric acid.

PROCESS. *Sampling.* In animal experiments, remove the muscle and organs immediately after slaughter, separate the visible fat and connective tissue from the muscle or organ tissue, and weigh each. Cut into small pieces, plunge in boiling water, and boil for 5 to 10 minutes to prevent enzymic hydrolysis. Separate the cooked meat from the broth and extract as directed below, combining both liquors.

Separate and weigh in like manner the parts of market cuts and grind the muscle or organ tissue to a finely divided pulp.

Solution. Weigh 100 g., add 200 ml. of water, heat slowly to boiling, and boil for 5 minutes with stirring. Allow the insoluble matter to settle and filter by decantation through an asbestos felt in a Büchner funnel. Add to the residue 150 ml. of hot water, boil, and decant as before, then repeat the operation a third time. Transfer the insoluble matter to the funnel and wash with small portions of hot water. Concentrate the filtrate to 25 to 30 ml., avoiding dryness.

Phosphotungstic Acid Precipitation. Cool the concentrated filtrate and transfer to a 100-ml. volumetric flask (keeping the volume below 70 ml.) containing 25 to 30 g. of *phosphotungstic acid* dissolved in 25 ml. of water, shake, let stand a short time for bubbles to escape, rinse with a little water, and make up to the mark. Shake vigorously, allow to stand until gas bubbles rise to the surface, and filter by suction through asbestos in a Büchner funnel. Test a few milliliters of the filtrate with *dry phosphotungstic acid.* If a precipitate forms, remove an aliquot to a volumetric flask, add an excess of *dry phosphotungstic acid,* make up to the mark, shake, allow to stand, filter, and test the filtrate as before.

Potassium Chloride Precipitation. When the precipitation of nitrogenous substances is complete, remove the excess of phosphotungstic acid by precipitation with *dry potassium chloride,* 0.076 parts being required for the complete precipitation of one part of photungstic acid. Filter and test a amount of the filtrate for creatinine by the addition of a few drops each of *picric acid* and *10% sodium hydroxide solution.*

Copper Reduction. Without delay, make up to a definite volume and determine reducing sugars in 25 ml. by the Munson and Walker or Allihn Method (Part I, C6*a*). Invert and determine total sugars as directed under the preceding method.

DEXTROSE

Folin and Wu Phosphotungstic-Molybdate Copper Reduction Colorimetric Method.[75] Although designed for blood analysis, this method may be employed for the determination of minute amounts of dextrose in tissue extracts. The Folin and Denis reagent is replaced by a reagent that reacts with cuprous copper in acid solution without the formation of a color with phenols.

APPARATUS. *Special Sugar Test Tubes* (Fig. 176).

REAGENTS. *Folin and Wu Reagent.* To 35 g. of $H_2MoO_4 \cdot H_2O$ and 5 g. of $Na_2WO_4 \cdot 2H_2O$, add 200 ml. of 10% NaOH solution and 200 ml. of water, then boil vigorously for 20 to 40 minutes. Cool, dilute to about 350 ml., add 125 ml. of 85% H_3PO_4, and make up to 500 ml.

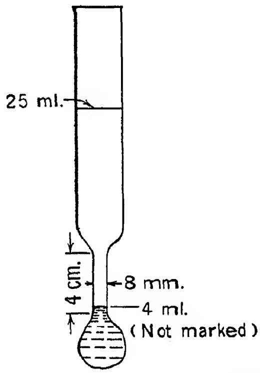
Standard Dextrose Solution, 1%, preserved with xylene or toluene. From this stock solution prepare as needed solutions containing (*a*) 1 mg. of dextrose per 10 ml. and (*b*) 2 mg. of dextrose per 10 ml.

Alkaline Copper Solution. Dissolve 40 g. of KOH in 400 ml. of water, add 7.5 g. of tartaric acid, and when dissolved add 4.5 g. of $CuSO_4 \cdot 5H_2O$. Mix and dilute to 1 liter.

PROCESS. *Copper Reduction.* Place in three sugar test tubes respectively: 2 ml. of the tungstic acid filtrate of the water extract

of the sample containing 70 to 400 mg. of dextrose per 100 ml., 2 ml. of sugar solution (*a*), and 2 ml. of sugar solution (*b*). To each tube add 2 ml. of *alkaline copper solution* and heat in the same boiling water bath for 6 minutes. Then cool in a cold water bath without shaking for 2 to 3 minutes.

Color Formation. Add to each tube 2 ml. of *Folin and Wu reagent.* When the cuprous oxide has dissolved (about 2 minutes for



Courtesy of J. Biol. Chem. 1920, 41, 372

FIG. 176. Folin and Wu Sugar Test Tube.

amounts of dextrose up to 0.8 mg.), dilute the blue solution to the 25-ml. mark, close with a rubber stopper, and shake well.

Color Comparison. Make the comparison after allowing to stand 5 minutes or 1 hour.

GLYCOGEN

Pflüger Alkali-Ethanol Copper Reduction Method.[76] This classical method, several times modified by Pflüger, in its latest form holds its own in competition with numerous modifications.

APPARATUS. *Polariscope.*

PROCESS. *Alkali-Ethanol Precipitation.* Heat 100 g. of the visible-fat-free comminuted sample with 100 ml. of *60% potassium hydroxide solution* in a 1.5-liter beaker on a boiling water bath for at least 3 hours, cool, add water to about 400 ml., followed by 800 ml. of *ethanol.* After allowing to settle overnight, pour off the liquid and add *66% ethanol containing 0.1% of saturated sodium chloride solution* to near the top of the beaker, stirring thoroughly. Repeat the decantation and addition of *66% ethanol* twice, thus obtaining the glycogen as a snow white deposit.

Dextrose Determination. Dissolve the glycogen in a small amount of hot water in a volumetric flask, add *acetic acid* to slight acid reaction, make up to the mark, and remove aliquots for polarization and for copper reduction by the Allihn Method (Part I, C6*a*).

CALCULATION. Use the equation: 1 g. of dextrose = 0.927 g. of glycogen.

I. Trowbridge and Francis Modification.[77] PROCESS. *Solution.* Mix 25 g. of the finely ground and thoroughly mixed sample, in a 400-ml. beaker, with 50 ml. of *potassium hydroxide solution,* cover with a watch glass, digest on the water bath for 2 hours with occasional stirring, and dilute to about 200 ml. with cold water.

Glycogen Precipitation. To the solution, add an equal volume of *ethanol,* cover with the watch glass, and allow the precipitated glycogen to settle for 10 to 12 hours. Decant the supernatant liquid onto an 18.5-cm. pleated paper and wash by decantation with *2 + 1 ethanol* about four times or until the glycogen is white or nearly so, then two or three times after transferring to the filter. Keep the funnel covered with a watch glass to prevent drying.

Resolution. Attach to the bottom of the funnel containing the washed glycogen a piece of rubber tubing closed by a pinch-cock, fill the funnel with warm water, cover with the watch glass, and let stand 2 or 3 hours or overnight. Open the pinchcock and allow the solution to pass through the filter into a beaker, then close the pinchcock and fill again with warm water. Allow to stand for 1 hour and filter as before. Continue the washing

with warm water until the filtrate, which at first is turbid, becomes perfectly clear.

Reprecipitation. Add to the solution twice its volume of *ethanol.* Allow to stand overnight to complete the separation. Collect the precipitated glycogen on a tared Gooch crucible, wash with *2 + 1 ethanol,* and dry in a boiling water oven to constant weight or, preferably, proceed as follows.

Copper Reduction. Filter on a paper, wash with *2 + 1 ethanol,* then dissolve in hot water as before, keeping the volume under 225 ml., and collect the solution in a 250-ml. volumetric flask. Hydrolyze by heating with 12.5 ml. of *hydrochloric acid* on a boiling water bath for 3 hours. Cool, nearly neutralize with *sodium hydroxide solution,* make up to the mark with water, and determine the copper reduction in an aliquot by the Munson and Walker or the Allihn method (Part I, C6*a*).

CALCULATION. Multiply the weight of dextrose by 0.9 to obtain the weight of glycogen.

II. Sahyun Charcoal Modification.[78] The modification, developed at Stanford University, employs active charcoal to aid in the separation of the glycogen by ethanol precipitation.

PROCESS. *Alkali Treatment.* Weigh into a 15-ml. graduated centrifuge tube 1 g. or less of the sample ground after freezing in *liquid air* and add *40% potassium hydroxide solution* to the 5-ml. mark. If larger amounts are used, to each gram in a 50-ml. centrifuge tube add 1 ml. of *60% potassium hydroxide solution* so that the final volume of the alkali mixture does not exceed 10 or 15 ml. Cover the tube with tinfoil and heat in a boiling water bath for 30 to 40 minutes, shaking every 5 or 10 minutes.

Ethanol-Charcoal Precipitation. Add 50 mg. of *active charcoal,* stir with a fine glass rod, then add twice the volume of *ethanol* and stir again. If any glycogen sticks to the glass rod, wash with as small an amount of hot water as possible into the tube and add twice that volume of *ethanol.* Centrifuge for 10 minutes. The charcoal together with absorbed glycogen collects at the bottom of the centrifuge tube. Discard the alkali-ethanol supernatant liquid.

Acid Hydrolysis. Add 5 ml. of hot distilled water or less, a strip of *litmus paper,* a few drops of *2 N sulfuric acid* sufficient to turn the *blue litmus paper* red, and then an equal volume of the *acid.* Heat the centrifuge tube in the boiling water bath for 2 hours, after which the hydrolysis is complete. Neutralize with *1.0 N or 2 N sodium hydroxide solution* and introduce quantitatively into a volumetric flask. Cool, dilute to the mark, mix thoroughly, and filter.

Determination of Dextrose. Treat the clear colorless solution as directed by Folin and Wu above, under Dextrose. The blue color obtained on adding the phosphomolybdic reagent does not change in intensity on standing 30 minutes.

CALCULATION. Multiply the weight of glucose found by 0.927 to obtain the weight of glycogen.

III. Simonovits Micro Modification.[79] The process, as practiced at the University of Budapest, is essentially the same as the Pflüger method except as to weights and volumes.

PROCESS. *Glycogen Precipitation.* Weigh 0.1 to 4 g. of the fresh comminuted tissue into a 15-ml. centrifuge tube, add 3 volumes of hot *60% potassium hydroxide solution,* and heat in a boiling water bath for at least 30 to 40 minutes. When the solution clears, cool, add 0.5 ml. of *saturated sodium chloride solution,* followed by 2 volumes of *ethanol,* mix thoroughly, and after allowing to stand 1 hour centrifuge 30 minutes, then pour off the supernatant liquid.

Glycogen Purification. Dissolve the precipitated glycogen in 3 ml. of hot water, add 0.5 ml. of *saturated sodium chloride solution,* again add 2 volumes of *ethanol,* and centri-

fuge. Again dissolve the glycogen in hot water (5 ml.), neutralize to *litmus paper* with *1% sulfuric acid*, and make up to volume in a 15-ml. volumetric flask. Filter through a dry paper and transfer 13 ml. of the filtrate to a centrifuge tube together with 1.5 ml. of *saturated sodium chloride solution* and 2 volumes of *ethanol*. Shake, after 5 hours or longer, centrifuge for 30 minutes, and pour off the supernatant liquid.

Hydrolysis. Dissolve the glycogen in 5 ml. of hot water, add 5 ml. of *2 N sulfuric acid*, cover with tinfoil, heat in a boiling water bath for 2 hours, cool, and neutralize with *2 N sodium hydroxide solution*. Make up to a suitable volume and determine the dextrose by the Allihn copper reduction method or the Bang iodometric method.

Calculation. Use the formulas: 0.0927 mg. of glycogen = 0.1 mg. of dextrose; 0.1 mg. of dextrose = 0.28 ml. of 0.01 *N* thiosulfate solution; also 0.1 ml. of 0.01 *N* thiosulfate solution = 0.0331 mg. of glycogen. Multiply the weight of glycogen by 15/13 to compensate for the dilution to 15 ml. and the removal of the 13-ml. aliquot.

IV. von Brand Sodium Chloride-Zinc Sulfate Micro Modification.[80] The distinctive feature is the reagent used in conjunction with absolute ethanol.

Reagent. *Sodium Chloride-Zinc Sulfate Reagent.* Dissolve 25 and 5 g. respectively of the salts in water and dilute to 100 ml.

Process. *Glycogen Precipitation.* Weigh into a centrifuge tube a suitable amount of the sample, add a suitable volume of *60% ethanol* (see other modifications for the proper proportions), and heat in a water bath until the tissue dissolves. Add an equal volume of water and 0.5 ml. of the *sodium chloride-zinc sulfate reagent* and mix by a current of air. Add 2 volumes of *absolute ethanol*, let stand 10 minutes, centrifuge, and wash the precipitate with 1 ml. of *90% ethanol*, then suspend the precipitate in 1 ml. of water and dissolve in *4.4% hydrochloric acid*. Filter into a 15-ml. centrifuge tube, rinsing the first tube twice with 1 ml. of water. Precipitate with *4% potassium hydroxide solution*, add 7 ml. of *absolute ethanol*, and centrifuge after 10 minutes. Dissolve again in *4.4% hydrochloric acid* and add 1 ml. additional.

Hydrolysis and Copper Reduction. Insert a cotton plug and hydrolyze by heating 3 hours in a boiling water bath. Cool, neutralize with *4% potassium hydroxide solution*, and determine dextrose in aliquots by copper reduction.

Calculation. Convert the weight of dextrose into the weight of glycogen by the factor 0.927.

Inositol

See also Part I, C9*d*.

Needham Carbon Combustion Gasometric Method.[81] Small amounts of inositol are determined by precipitation by ethanol from a clarified solution, combustion in a stream of oxygen, absorption of the carbon dioxide by barium hydroxide, liberation from the latter, and measuring.

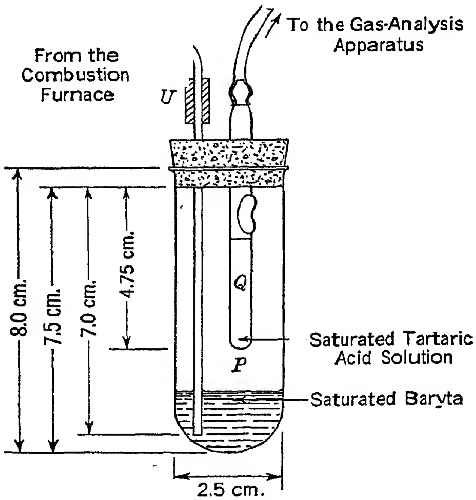
Apparatus. A train consisting of (1) *combustion furnace, quartz combustion tube* and the usual bulbs and tubes for the removal of carbon dioxide from the stream of oxygen and the drying of the gas, (2) *absorption apparatus* (Fig. 177), and (3) *Haldane gas-measuring apparatus* (Fig. 178).[82]

Process. *Extraction.* Remove visible fat from 6 kilos of the sample (such as meat), treat with 750 ml. of *acetone*, allow to stand overnight, and filter. Because of the water in the sample, the strength of the acetone is only about 10 or 12%.

Defecation. Add *neutral (normal) lead acetate solution* followed by *basic lead acetate (lead subacetate) solution* and remove the precipitate by filtering and washing. From the filtrate, precipitate the excess of lead salts by a stream of *hydrogen sulfide*, then filter and wash. Evaporate the filtrate under diminished pressure to 20 to 30 ml.

Ethanol Precipitation. To the concentrated solution, add 300 ml. of *ethanol,* allow to stand overnight, collect the precipitate on a Gooch crucible, and dry by sucking air through the crucible for 10 minutes. Transfer the crucible and contents to a flask, shake with 60 ml. of water to dissolve the inositol, filter into a 100-ml. volumetric flask to remove the asbestos, and make up to the mark.

Combustion. Measure with an accurate



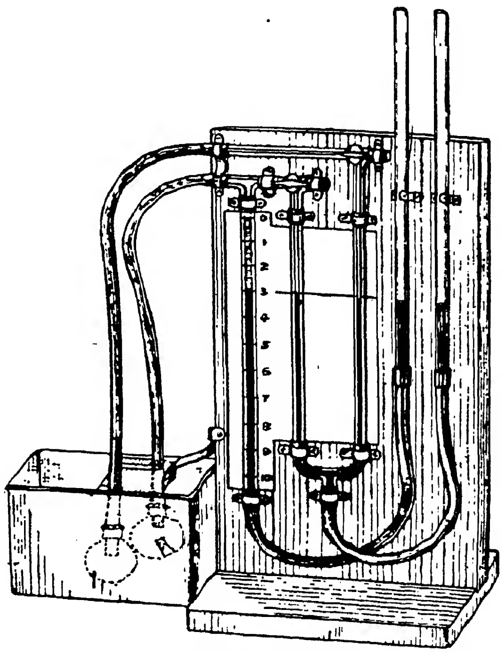
Courtesy of Biochem. J. 1923, **17**, *432*

Fig. 177. Needham Absorption Apparatus.

micro buret an aliquot of the solution, containing 30 to 40 mg. of inositol, into a boat, introduce into the quartz tube, and carry out the combustion in a stream of *oxygen.*

A wash bottle containing *sulfuric acid,* a tube containing *soda lime* or sticks of *potassium hydroxide,* and a wash bottle containing *baryta water* serve to dry and remove carbon dioxide from the oxygen before it reaches the substance. A bulb removes moisture condensed from the carbon dioxide which may be drawn off by a tap. Water vapor is absorbed by a sulfuric acid bulb and a calcium chloride tube; cooling is effected by a long coil and further protection from heat by an asbestos screen.

A two-way tap permits direction of the carbon dioxide gas into an index-bubbler or



Courtesy of J. Path. Bact. 1920, **23**, *444*

Fig. 178. Haldane Gas-Measuring Apparatus.

through the absorption apparatus where it is taken up by the barium hydroxide solution, any gas collecting in the bottom of the cooling coil being washed out by a rapid stream of oxygen. The rubber collaret (*U*) permits grasping by tongs during shaking to hasten evolution of gas.

The outer tube (*P*) contains 5 ml. of *concentrated barium hydroxide solution* and the inner tube (*Q*), with a side outlet, contains 1 ml. of *tartaric acid solution.* The tartaric acid solution is emptied into the barium hydroxide solution for the final evolution of the

gas which in turn is measured in the Haldane apparatus.

Carbon Dioxide Liberation and Measurement. After the combustion is finished, shake the apparatus so as to deliver the tartaric acid solution into the *barium hydroxide solution,* thus liberating the gas. Measure the volume of the gas in the 1-ml. buret, graduated to 0.01 ml. but reading to 0.002 ml., of the Haldane apparatus or its equivalent.

This stage in the procedure is in substance a micro elaboration of the classical volumetric method of determining carbon dioxide in carbonates which, as applied to baking powder, is described in Part II, K2.

Blank. Make a blank determination with the empty boat or the boat filled with water. Needham obtained blanks ranging from 0.070 to 0.102, average 0.081 ml., which illustrate the delicacy of the measurement.

CALCULATION. Reduce the volume of gas to the dry basis at 0° and 760 mm. pressure. The calculation is facilitated by reference to Haldane's *Methods of Gas Analysis.*[83]

Theoretically, 1 ml. of carbon dioxide gas = 1.337 mg. of inositol or 1 mg. of inositol = 0.758 ml. of carbon dioxide gas.

FIBER

Meat, although made up of muscle fibers and fibrous connective tissue, contains no fiber analogous to that of vegetable products. In mixtures of meat with cereal and vegetable products, such as dog biscuit, follow the usual method (Part I, C2*e*).

RIBOFLAVIN

(Vitamin B_2)

See also Part I, C10.

Van Eekelen and Emmerie Oxidation Colorimetric Method.[84] The feature characterizing this method is the measurement of the color absorption of riboflavin, not the color value of lumiflavin formed from riboflavin as in the Kuhn, Wagner-Jauregg, and Kaltschmitt method, nor the fluorescence of riboflavin as in the von Euler and Adler method. Foreign colors are destroyed by oxidation with permanganate. The method was designed for liver.

APPARATUS. *Photometer* (Zeiss), with screen S 47 and 3-cm. cell.

PROCESS. Carry out all manipulations in red light.

Extraction. Grind 10 g. of liver with five to ten times that amount of *75% methanol,* extract for at least 24 hours at 37°, or boil for about 10 minutes, and filter.

Oxidation. Treat 20 ml. of the filtrate with 2 ml. of *glacial acetic acid* and 2 ml. of *saturated potassium permanganate solution,* allow to stand 10 minutes, then add 1 ml. of *hydrogen peroxide.*

Color Reading. Measure the color absorption of the yellow-green solution in a 3-cm. cell of the photometer, using screen S 47.

Van Duyne Pepsin Digestion Fluorometric Method.[85] Finding that incubating or refluxing liver with methanol or/and ethanol of different concentrations, with or without hydrochloric acid, extracted only 43 to 73% of the riboflavin and that 60 to 80% acetone produced solutions with a deep blue fluorescence that masked the yellow-green fluorescence of riboflavin, Van Duyne, of Columbia University, turned to pepsin digestion. The method gives results on liver, kidney, and heart muscle agreeing closely with those by the biological method, but is not applicable to skeletal muscle because of the low content.

APPARATUS. *Klett Fluorimeter;* Type H-4 mercury vapor lamp. Corning glass filters No. 584 are placed between the lamp and the fluorescent solutions and Nos. 440 and 401 between the latter and the photronic cells.

Leeds and Northrup Mirror Galvanometer.

REAGENT. *Pepsin Solution,* 0.3% in 0.2% HCl, preserved by a few drops of chloroform.

PROCESS. Freeze 0.5 to 2.0 g. of the tissues

with *solid carbon dioxide,* grind with *sand,* and digest for 20 hours at 37° with 25 to 50 ml. of *0.3% pepsin solution.* Boil the digestion mixture, decant on a filter, and wash by decantation. Dilute to a uniform concentration of pepsin and acid and a riboflavin concentration of 0.2 to 0.6 γ/ml.

Fluorescence Reading. Determine the fluorescence of the clear, faintly yellow solution directly in the Klett instrument illuminated with the 3650 Å line of the spectrum. Prior to a final estimation, shield the solution from light.

CALCULATION. Compare with a graph showing (1) a standard curve representing the intensity of fluorescence of riboflavin solutions of known concentration in terms of gammas per milliliter of riboflavin solution as standard and (2) a curve plotted for riboflavin in pepsin and hydrochloric acid solution read against the standard. The abscissas are negative logarithms of concentrations of 0 to 1.0 γ/ml. of riboflavin, the ordinates potentiometric readings 0 to 500 ml. The first curve is a straight line between 0.9 and 0.1 γ/ml., the second is not linear below 0.2 γ/ml.

EXAMPLES. Rat liver 30.5 to 40.8; rat kidney 28.7 to 34.8; rat heart muscle 26.6 to 28.3 γ/g.

NICOTINIC ACID

Swaminathan Aniline-Cyanogen Bromide Colorimetric Method; Melnick and Field Modification. See Part I, C10.

McIntire, Waisman, Henderson, and Elvehjem [86] employ the modification, with suitable amendments, in the examination of meat.

Bandier Metol-Cyanogen Bromide Colorimetric Method. See Part I, C10.

Dann and Handler,[87] in applying the method to meat, extract the sample with hot *dilute hydrochloric acid* and decolorize with *Lloyd's reagent* and *lead hydroxide.* The presence of color in the extracts renders extrapolation inaccurate and decolorization by charcoal introduces an error.

NICOTINAMIDE

Karrer and Keller Dinitrochlorobenzene Colorimetric Method.[88] All pyridine derivatives give with dinitrochlorobenzene (2,4-$C_6H_3(NO_2)_2Cl$) pyridinium salts which are decomposed with alkali with the formation of yellow-red derivatives of glutaconaldehyde. Of the group only nicotinamide is usually present in animal products; plant products, however, may contain pyridine alkaloids.

APPARATUS. *Leifo Step-Photometer* (Fretwurst and Maennchen: *Photometrische Bestimmungen in der medizinischen Chemie mit dem Leifo-Photometer,* Wetzlar).

PROCESS. *Extraction.* Boil a suitable amount of the sample (e.g., 300 g. of beef liver) for 1 hour with *0.1 N potassium hydroxide solution,* neutralize carefully with *hydrochloric acid,* evaporate to dryness *in vacuo,* and pulverize. Extract the powder with *benzene* and evaporate the extract to dryness. Fuse the residue for about 1 hour at 90° with about 4 parts of *dinitrochlorobenzene* for each part of nicotinamide. Cool the melt, transfer to a separatory funnel with the aid of *ether* and water, and add 10 ml. of water in addition. Shake, remove the aqueous layer and treat several times with *ether* to remove the excess of the reagent, then wash the combined ether extracts with water and add to the aqueous liquid. Pour the combined aqueous extracts into a volumetric flask, warm slightly to remove traces of dissolved ether, and dilute to the mark. Remove by filtration any of the dinitrochlorobenzene which may have precipitated in the water.

Color Formation. Transfer an aliquot of the clear aqueous solution to the colorimeter tube and add 1 or 2 drops of *20% potassium hydroxide solution.* The color forms immediately but soon begins to fade.

Color Reading. Without delay read the color value in a Leifo step-photometer. (A Rosenheim-Schuster tintometer is less satisfactory since the red and the yellow color do not diminish equally in dilution.

CALCULATION. Compare with a curve plotted with concentrations of nicotinamide ranging from 5×10^{-1} to 1×10^{-3} mg. per ml.

Colors

See Part I, C11 and C12.

Ash

See Part I, C2*f*, C8*a* and *b*.

Ignite with sodium carbonate and determine chlorine either gravimetrically or by the Volhard Volumetric Method (Part I, C8*a*).

Multiply the percentage of chlorine by 1.6486 to obtain the corresponding percentage of sodium chloride. Since unsalted meat contains about 1% of ash, of which only about 0.05% is chlorine, and corned meat contains from 2 to 9% of ash, the greater part of which is common salt, calculation by the factor gives a close approximation of the truth.

Chemical Preservatives

In addition to salt and wood smoke, the time-honored and universally allowed antiseptics, sulfurous acid, combined as sulfites, and boracic acid, both free and combined as borax, at one time were quite generally employed in curing meats and fish, as well as in preserving sausage, Hamburg steak, and other comminuted animal products. Aside from hygienic consideration, these are better suited for the preservation of animal products than benzoic acid, free or combined as sodium benzoate, and salicylic acid which are preferred for vegetable and fruit products.

Sodium benzoate is, with restrictions, an allowed preservative for fish and meat products in the United States, notwithstanding the conclusions of Wiley, an opponent of all chemical preservatives, that, if any is allowed, salicylic acid is less objectionable than benzoic acid, and the belief of Chittenden, a member of the Federal Referee Board, based on his own experimental evidence and as once expressed to one of the writers, that the harmfulness of boracic acid has been exaggerated.

Since sulfites not only preserve chopped meat from spoilage but also aid in preserving the natural color, the butcher has been loath to abandon their use in compliance with legal regulations.

See Part I, C13, for methods of detection and determination.

2. SAUSAGE

In the United States the term sausage without qualification is applied to an uncooked pork product seasoned as permitted by the U. S. Standard with "herbs, spice, common salt, sugar, dextrose, a sirup, water." In addition to this standard American product, liver sausage, Bologna sausage, Hamburger sausage, headcheese, and a great variety of sausage made according to foreign recipes and often known under foreign names is sold in food shops.

Moisture

Official A.O.A.C. Methods.[89] Weigh 10 g. of the sample into a tared weighing bottle containing a short glass rod flattened at one end. Remove 2.5 to 3 g. for nitrogen determination and reweigh, then spread the remainder over the sides and bottom. Dry at 101 to 102° for 16 to 18 hours, or at 120 and 130° for 2 to 3 hours, or else until there is no significant loss during a subsequent 1- to 2-hour period. Drying *in vacuo* at 95 to 100°

or at room temperature over sulfuric acid are alternate methods. Water in excess of that calculated by multiplying the percentage of protein by 4 is regarded as added.

NOTE. The factor 4 is in accord with the results of Robison [90] who found that the ratio of water to protein in sausage made from the fresh carcass ranged from 3 : 1 to 3.6 : 1. Robison dried 100 to 500 g. of the sausage first at 70 to 90° over a radiator for 10 to 15 hours, ground, and weighed the material, then dried 2 to 5 g. of this subsample in a boiling water oven, employing a stream of *hydrogen* in exact work. If rich in fat, he performed the first drying on a sieve over a vessel to collect the fat and then dried portions of fat and lean as above.

Although the fat may sustain some oxidation during direct drying, the extent of the error so caused is ordinarily negligible and doubtless less than that due to losses during sampling and grinding.

Starch and Glycogen

Cereal flour or other starchy material is often an ingredient of sausage and other chopped meat preparations. Formerly attempts were made to detect horse meat by the high glycogen content, but it is now known that horse meat does not necessarily contain more glycogen than other meats and that the loss during aging is rapid and very considerable in all meats. Bovine liver may contain over 3%.

If the iodine test shows that the sample contains starch beyond that occurring in spices, the result by the following method represents starch and glycogen; the latter, however, in ordinary meat is present in relatively small amount and may often be ignored. If a separation and determination of the two is desired, combine this method with the Baur and Polenske Method below. On the other hand, if there is no added starchy filler, the result represents glycogen alone.

Mayhofer Alkali-Ethanol Gravimetric Method.[91] REAGENT. *Ethanolic Potassium Hydroxide Solution.* Dissolve 80 g. of KOH in 1 liter of 90% by volume ethanol.

PROCESS. *Alkali Digestion.* If a large amount of fat is present, remove by mechanical means or by *ether* extraction from 50 g. of the sample. Mix the residue, or 50 g. of the original sample directly, with 150 ml. of *ethanolic potash* and heat for 30 minutes on a boiling water bath with stirring.

Precipitation. To the hot liquid, add 100 ml. of *50% ethanol,* cool, filter by suction on paper, and wash first with 30 ml. of *ethanolic potash* at 50° and then with cold *90% ethanol* until no precipitate forms in the washings when mixed with a few drops of *dilute hydrochloric acid.*

Reprecipitation. Wash the residue into a 110-ml. volumetric flask with 50 ml. of *1.0 N aqueous potassium hydroxide solution* and heat on a boiling water bath for 30 minutes to dissolve the precipitated carbohydrate matter. Cool, add *glacial acetic acid* to acid reaction, make up to the mark, mix, and filter through dry paper. Pipet 100 ml. of the clear filtrate into a beaker, add 150 ml. of *absolute ethanol,* and allow the starch and glycogen to settle. After 12 hours, collect the precipitate on a tared Gooch crucible or filter paper, wash with *70% ethanol* until a small portion of the filtrate on evaporation leaves no residue; then wash successively with a little *absolute ethanol* and *ether.* Heat the precipitate at about 40° to remove the ether, then dry at 100° to constant weight. Ignite a portion of the precipitate and correct for ash.

CALCULATION. Multiply by 2.2 to obtain the percentage of starch or glycogen or both.

A determination of starch in a portion of the weighed remainder of the precipitate by hydrolysis and copper reduction is desirable.

I. Price Copper Reduction Modification.[92] Bigelow [93] slightly modified the Mayhofer method, but still retained the steps of precipi-

tation of starch and weighing as such, which Price found impracticable.

Process. *Alkali Digestion.* Weigh 200 ml. of the sample into a beaker and digest on the steam bath with 75 ml. of *8% potassium hydroxide solution in ethanol* for 30 to 45 minutes or until all the meat has dissolved.

Ethanol Precipitation. Add an equal volume of *ethanol*, cool, let stand at least 1 hour, and filter through a thin layer of asbestos in a Gooch crucible. Wash twice with warm *4% potassium hydroxide in 50% by volume ethanol* and twice with warm *50% ethanol*, retaining as much as possible of the precipitate in the beaker until the last washing.

Acid Hydrolysis. Place the crucible in the original beaker, add 40 ml. of water and 25 ml. of *sulfuric acid* with stirring. After 5 minutes, add 40 ml. of water, heat just to boiling with constant stirring, and transfer to a 250-ml. volumetric flask. Add 2 ml. of *20% phosphotungstic acid solution*, cool to room temperature, make up to the mark, and filter through starch-free paper.

Copper Reduction. Pipet 100 ml. of the filtrate into a 200-ml. volumetric flask, neutralize with *1 + 1 sodium hydroxide solution*, dilute to the mark, filter, and determine the dextrose in an aliquot by the Munson and Walker method.

Calculation. Obtain the weight of starch corresponding to that of dextrose by the factor 0.9.

II. Rask-McVey Cold Acid Digestion Modification.[94] Rask took advantage of the solubility of starch in 21% hydrochloric acid at room temperature; McVey developed the following rapid procedure that is accurate in the presence of hemicelluloses.

Process. *Alkali Digestion.* Weigh 10 g. of the well-mixed sample into a 150-ml. beaker, add 50 ml. of *8% ethanolic potassium hydroxide solution*, break up lumps, and heat on the steam bath for 30 to 45 minutes or until the material has been completely disintegrated. Add 40 to 50 ml. of *ethanol*, then with stirring 1 g. of *Filter-Cel*, and let stand a few minutes.

Filter through a Gooch crucible fitted with a paper disk, wash well with *ethanol*, and suck dry. Transfer the material and paper to the original beaker by tapping the crucible and wiping with filter paper.

Cold Acid Digestion. Add *5.7 N hydrochloric acid* and stir to a creamy consistency, then transfer to a 100-ml. volumetric flask by means of 50 to 60 ml. of *5.7 N hydrochloric acid*. Stopper, shake vigorously for 3 to 5 minutes, and let stand 15 minutes, or, better, shake for 10 minutes in a mechanical shaker. Dilute to volume with water, mix, and filter through a mat of asbestos in a Gooch crucible.

Ethanol Precipitation. Pipet 50 ml. of the filtered solution into 115 ml. of *ethanol* contained in a 250-ml. beaker and stir vigorously, then before the precipitated starch has coagulated or settled pour the Filter-Cel from a Gooch crucible previously tared together with a thin asbestos mat and the Filter-Cel (two-thirds full) after drying at 100°. Stir well, allow to settle for 5 minutes, and collect the Filter-Cel and precipitate on the Gooch crucible. Wash with *ethanol*, dry overnight at 100° or for 1.5 hours at 125°, cool in a desiccator, and weigh quickly.

Calculation. Multiply the weight of starch by 20 and by 1.27 or other appropriate factor to obtain respectively the percentage of starch and corn flour or other cereal flour.

Baur and Polenske Ammonium Sulfate Gravimetric Method.[95] Reagent. *Iodine Solution.* Dissolve 0.5 g. of iodine and 2 g. of KI in 150 ml. of water.

Process. *Starch Separation.* Weigh 0.3 to 0.5 g. of the material consisting of starch and glycogen, obtained by the Mayhofer Method above, and dissolve in 30 to 40 ml. of water. Add twice the volume of *saturated ammonium sulfate solution*, allow to stand 30 minutes, and separate the dissolved glycogen from the precipitated starch by filtration.

Before washing the precipitate, mix a few drops of the filtrate on a spot-plate with a few drops of *iodine solution*. If a blue color appears, add *saturated ammonium sulfate solution* to the whole filtrate and allow to settle for at least 30 minutes and continue the filtration, adding the second precipitate to the first. If, on the other hand, the color in the test is red-violet by reflected and Bordeaux red by transmitted light, the first precipitation is complete.

Starch Determination. Wash the precipitate 3 times with *half-saturated ammonium sulfate solution*, then dissolve in *1.0 N sodium hydroxide solution*, using the first portions to clean the beaker, and wash finally with water until the volume of the filtrate is about 100 ml.

To the opalescent filtrate, add *acetic acid* until the alkali is neutralized and the solution is slightly acid, then precipitate the starch with 150 ml. of *absolute ethanol*, and allow to settle 12 hours. Filter on a tared Gooch crucible or filter paper, wash first with *50% ethanol*, then with *absolute ethanol*, and finally with a little *ether*. Heat at about 40° to remove the ether and dry at 100° to constant weight.

Glycogen Determination. Subtract the weight of starch thus obtained from that of starch plus glycogen, thus obtaining the weight of glycogen or, if preferred, dilute the filtrate from the starch with 3 to 4 volumes of water, precipitate with an equal volume of *95% ethanol*, filter after 12 hours, wash with *50% ethanol*, and dissolve in a small amount of water. Again precipitate the glycogen in the opalescent solution, filter, wash, dry, and weigh as before.

Lactose

See also Part I, C6*a*, and Part II, G1.

Cole Lactosazone Test.[96] The test developed by Cole depends on the reaction of lactose with phenylhydrazine to form lactosazone. It was designed for testing urine.

McVey and McMillin Modification for Sausage.[97] In the adaptation of the Cole test, as a means of detecting dried skim milk in sausage and other comminuted products, developed in the U. S. Bureau of Animal Industry, the lactose from an aqueous extract is adsorbed by activated charcoal.

Process. *Extraction.* Mix in a beaker 25 g. of the finely divided sample, 1 to 2 g. of *powdered calcium carbonate*, and 50 ml. of warm water. Break up lumps with a rod and boil a few minutes. Centrifuge and separate the fat either in a separatory funnel or by filtering through a wet pleated paper. Clarify 25 to 30 ml. of the extract by shaking with 10 ml. of *alumina cream* and filtering after a few minutes through a wet paper.

Charcoal Adsorption. Shake vigorously in a 125-ml. Erlenmeyer flask 25 ml. of the filtrate with 1 g. of *adsorbent charcoal*, boil a few seconds, cool, and let stand 10 minutes with frequent shaking. Filter through a disk of filter paper in a Gooch crucible, wash with 2 to 3 ml. of water, and suck dry. Transfer the charcoal to a 125-ml. Erlenmeyer flask and boil for about 10 seconds with 10 ml. of water and 1 ml. of *glacial acetic acid*.

Lactosazone Formation. Filter the hot mixture through a small paper into a large test tube containing 0.5 g. of *phenylhydrazine hydrochloride* and 2 g. of *sodium acetate*. Heat the tube in a boiling water bath for a few minutes, then shake and heat 45 minutes longer. Filter while hot and let stand at room temperature at least 1 hour. If no crystals form after 1 hour, stopper and let stand 18 hours. If no crystals form, the test is negative. Examine the crystals under a cover-glass with a magnification of 100 to 150 diameters.

Crystalline Formation. The lactosazone crystallizes in characteristic "hedgehog" clumps with projecting spines, which usually terminate in long hair-like appendages. Recrystallization is not usually satisfactory.

McVey and McMillin Selective Fermentation Method.[98] The results obtained by this method serve not merely for the detection of skim milk in sausage, but also for the quantitative estimation.

Reagents. *Washed Yeast Suspension.* Macerate 2 cakes of compressed yeast with 150 ml. of water. Centrifuge for 5 minutes and reject the aqueous layer. Repeat the operation four times until the supernatant liquid is practically clear. Finally prepare a suspension in water and dilute to 100 ml. Store at about 4°, shake well for use, and reject after 2 weeks.

Benedict Reagent. (1) Dissolve 16 g. of crystallized copper sulfate in 125 to 150 ml. of water. (2) Dissolve 150 g. of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 130 g. of anhydrous sodium carbonate, and 10 g. of sodium bicarbonate in 650 ml. of hot water. Mix (1) and (2), cool, dilute to 1 liter, and filter.

Process. *Extraction and Clarification.* Prepare a homogeneous mixture of 10 g. of the sample and 80 ml. of warm water. Warm on the steam bath for 30 to 60 minutes with occasional stirring, transfer to a 100-ml. volumetric flask, and cool in a cold water bath. Add 2 ml. of *hydrochloric acid* and 5 ml. of *20% phosphotungstic acid solution,* make up to volume with water, add 5 ml. more to correct for the volume of the insoluble matter, mix, and filter. Pipet 40 ml. of the filtrate into a 50-ml. volumetric flask, neutralize with *sodium hydroxide solution,* and dilute to volume.

Yeast Treatment. Add 40 ml. of the neutralized solution to a tube containing the deposit remaining after centrifuging 5 ml. of the washed *yeast suspension* and draining off the water layer. Shake vigorously, let stand with occasional shaking for 1 hour, then whirl again, and decant the clear extract.

Copper Reduction. Pipet into 20 ml. of *Benedict reagent,* in a 300-ml. Erlenmeyer flask, 10 ml. of the above extract, or a volume containing 5 to 25 ml. of lactose diluted to 10 ml. Heat to boiling in 3 to 5 minutes, continue for exactly 3 minutes, then cool quickly under the tap. If no appreciable reduction has taken place, reject the solution and repeat the operations with another 10-ml. aliquot with the addition of 1 ml. of *standard 1% lactose solution* and 20 ml. of *Benedict reagent* and correct as directed below.

Titration by Modified Scales Method. Dilute the cooled solution containing the reduced copper with 100 ml. of cold water and add slowly with shaking 10 ml. of *24% acetic acid,* then with constant shaking add a measured volume of approximately *0.04 N iodine solution,* which is at least 5 ml. in excess of the quantity necessary to dissolve the cuprous oxide to a clear, dark green solution. After a few minutes, add 20 ml. of *20% phosphoric acid,* and titrate the excess of iodine solution with *0.04 N sodium thiosulfate solution,* using *starch solution indicator.*

Determine the relative standard as follows: Boil for 3 minutes 20 ml. of *Benedict reagent* with 10 ml. of water, and cool quickly. Add 100 ml. of water, 10 ml. of the *acetic acid,* 10 ml. of the *iodine solution,* and 20 ml. of the *phosphoric acid solution* and titrate with the *thiosulfate solution.*

Also determine the lactose factor for the iodine solution by repeating the procedure, adding to the solution before boiling 1 ml. of *standard 1% lactose solution.*

Calculation. Obtain the per cent of dried skim milk (S) by the formula:

$$S = \frac{KV_x}{5A}$$

in which K is the number of milligrams of lactose represented by each milliliter of the iodine solution, V_x is the milliliters of iodine solution absorbed (corrected for the volume required to dissolve the Cu_2O from the reduction of 10 mg. of lactose if added), and A is the weight of meat represented by the aliquot used. When the aliquot is 10 ml., $A = 0.8$ g.

Calcium Oxide

McVey and McMillin Oxalate-Permanganate Volumetric Method.[99] Normal comminuted meat products were found to contain only 0.017 to 0.032% of calcium oxide, but dried skim milk contained 1.72 to 1.97, average 1.84%. The U. S. Department of Agriculture regulation limits the amount of skim milk powder added to meat products to 3.5%, which amount increases the amount of calcium oxide four- to five-fold. Due regard, however, must be given to soy bean flour containing 0.40 to 0.60 and tripe containing as high as 0.15% of calcium oxide.

Process. *Incineration.* Press firmly against the bottom and sides of a tared platinum or porcelain dish 25 g. of the finely ground sample and reweigh. Place in an unheated muffle furnace and slowly raise the heat with free access to air. When the contents of the dish have ignited, open the door, then, when the burning ceases, finish the combustion at 500 to 600°. Add 5 ml. of *hydrochloric acid,* evaporate to dryness on the steam bath, dissolve the residue in 15 to 20 ml. of warm *1 + 4 hydrochloric acid,* filter into a 125-ml. Erlenmeyer flask, and wash with 30 ml. of hot water.

Oxalate Precipitation. Add 10 ml. of *saturated ammonium oxalate solution* and heat to boiling, then remove the heat and add 1 to 2 drops of *methyl red indicator,* followed with constant shaking by *1 + 1 ammonium hydroxide* until a permanent turbidity appears or until the solution is neutral. Boil, further neutralize if necessary, and let stand several hours. If a precipitate forms which remains while the solution is distinctly acid to methyl red, the test is positive; if no turbidity appears until after the neutral point has been nearly or quite reached, the test is negative.

Collect the precipitate on a filter and wash until oxalates are removed, then pierce the tip of the paper and wash the precipitate into the precipitation flask with warm water. Add 10 ml. of *1 + 4 sulfuric acid* and water to a total volume of about 60 ml. Finally heat to 90° and titrate to a permanent pink color with *standard 0.05 N potassium permanganate solution,* then add the filter paper and complete the titration.

Calculation. Obtain the per cent of calcium oxide (C) and calculate the per cent of dried skim milk (S) by the formula

$$S = \frac{C - 0.024}{0.0184}$$

Soy Bean Flour

Hendrey[100] (Spencer Kellogg and Sons, Buffalo) bases the determination of soy bean flour in sausage on the usual presence of considerable amounts of "soluble sugars" (11.80 to 14.94%), "insoluble sugars" (11.43 to 13.32%), and "nonfermentable sugars" (9.22 to 10.31%) in soy bean flour and the small amount of all three in meat powder (0 to 0.35, 1.31 to 1.41, and 0 to 0.10%) and ground meat (0.045 to 0.204, 0.043 to 0.075, and 0.004 to 0.018%), all calculated as invert sugar. The procedures employed are modifications of the usual processes.

Colors

See Part I, C11 and C12.

Preservatives

See Meat above, also Part I, C13.

3. MEAT EXTRACTS

The liquor resulting from the boiling of meat in the preparation of canned meat, which in the early days of the industry was wasted, in modern times has been utilized for the manufacture of meat extracts.

Composition. It has been stated by one manufacturer that 1 pound of extract is the product of about 32 pounds of lean meat free from bone and fat, but analyses of American

commercial extracts indicate that the soluble constituents in 1 pound of solid extract, although extremely variable, represent more nearly the soluble non-coagulable constituents of 10 pounds of lean meat and 1 pound of liquid extract about half that amount. Doubtless large pieces of meat on boiling sustain much smaller losses of soluble matter than when finely comminuted meat is treated with cold water in the determination of soluble nitrogenous matter. When made from corned beef liquor, the extract of necessity contains a considerable amount of salt and appreciable amounts of nitrate and nitrite.

Solid Meat Extracts, as analyzed by Bigelow and Cook[101] contained as follows: moisture 12.39 to 26.50; nitrogen: total 6.02 to 9.07, ammonia 0.20 to 0.71, insoluble plus coagulable 0.06 to 0.48, proteose 0.77 to 2.02, peptone 1.09 to 2.68, meat base 3.05 to 4.21, creatine plus creatinine 0.75 to 1.24, purine base 0.03 to 0.52, other meat base 2.08 to 2.65; fat 0.43 to 1.30; acid as lactic 4.15 to 8.42; ash: total 20.46 to 31.68, sodium chloride (Cl $\times$ 1.6486) 3.11 to 18.32; total phosphoric acid 2.29 to 4.55, organic phosphoric acid 0.18 to 0.61, inorganic phosphoric acid 1.79 to 4.06; undetermined 5.89 to 21.04%.

Liquid Meat Extracts contained 49.94 to 68.97% of moisture and correspondingly less of the solid constituents.

Micko[102] demonstrated that the purine of meat extract present in largest amount is hypoxanthine, whereas xanthine and adenine occur in appreciably smaller amounts and guanine is absent, although present in meat.

Yeast Extracts. *Micko*[102] found 0.30 to 1.14% of purine base nitrogen, but no creatine or creatinine. The chief purine was adenine, the other purines being guanine, hypoxanthine, and xanthine in decreasing order of content.

Analytical Methods. The methods of analysis for true meat extracts are essentially the same as those for the water-soluble constituents of fresh meat, but the presence of foreign constituents, such as glycerol, sugar, and gelatin, introduce complications.

Sample

Rub up in a mortar to a homogeneous paste solid extracts and bouillon cubes. Shake fluid extracts and juices so as to bring into suspension any sediment in the container.

Moisture

Weigh 2 to 10 g. of the sample into a flat-bottom dish 5 cm. in diameter, dry on a steam bath until no visible liquid remains, and proceed with the drying as directed under Meat above. In the official method for pasty extracts, sand, pumice stone or asbestos is specified.

Insoluble Matter

Solid meat extracts contain on an average ten times as much soluble organic solids as meat, coagulable proteins having been largely removed in manufacture. Proceeding according to the Emmett method of solution for meat, 2.5 g. are approximately equivalent to 25 g. of fresh meat, but a larger amount, such as 8 to 10 g. as directed for the A.O.A.C. Tentative Method for the determination of insoluble nitrogen, may be used. The bulk of the material is readily soluble and the content of coagulable nitrogen is small. Use about double these amounts for fluid extracts and bouillon cubes.

Stir well with several portions of cold water and filter through a weighed paper into a 500-ml. volumetric flask. Finally wash with water until the filtrate reaches the mark. Dry the paper and insoluble matter in a boiling water oven and weigh.

Insoluble Nitrogen

Determine nitrogen in the insoluble matter or obtain the insoluble nitrogen by difference,

subtracting the soluble nitrogen from the total nitrogen.

Soluble Nitrogen

Use aliquots of the filtrate from the insoluble matter, made up to 500 ml., for the determination of the individual soluble nitrogenous constituents.

If insoluble matter is not determined, an alternate procedure is to weigh the charge into a 500-ml. volumetric flask, add cold water to the mark, shake until solution is effected, and filter through a dry paper.

In either case, determine *total soluble nitrogen* in an aliquot of 25 to 50 ml. and measure two 100-ml. aliquots: (1) for subsequent determination of *coagulable proteins,* then in the filtrate *proteoses* by the zinc sulfate method, and (2) for determination of *coagulable proteins,* then in the filtrate *proteoses and peptones* by the tannin-salt method and, in the second filtrate, *meat bases and amino acids;* also measure an aliquot (3) of 150 ml. for the determination of *creatine* and *creatinine.*

Proceed with the determinations in aliquots (1), (2), and (3) as directed under Meat, adjusting subsequent aliquots to meet the conditions.

Proteoses and Peptones

Schjerning Tannin-Salt Method Modified by Bigelow and Cook.[103] Details are given under Meat, above. The method yields only approximate results, one of the errors being the presence of creatine in the tannin-salt precipitate.

Creatinine

Folin-Benedict Picric Acid Method. See Meat above.

I. Bigelow and Cook Modification. See Meat above.

II. Sudendorf and Lahrmann Modification.[104] At the Hamburg Hygienic Institute the following modification is employed.

Apparatus. *Duboscq Colorimeter.*

Process. *Acid Conversion.* Weigh 10 g. of a meat extract or a proportionately larger amount of bouillon cubes into a 100-ml. flask, digest with water, make up to the mark, and filter through a dry paper to remove fat and insoluble matter. Pipet 10 to 20 ml. of the clear solution into a porcelain dish, add 10 ml. of *1.0 N hydrochloric acid,* and evaporate on the water bath to dryness (2 hours). Take up with water, neutralize accurately to *litmus* with *0.5 N sodium hydroxide solution* (spot-plate method), transfer to an Erlenmeyer flask, and dilute to 75 ml.

Clarification. To the solution, add *1% potassium permanganate solution* dropwise to a brown-red color suggesting that of Malaga wine. If the sample contains no salt, use a *permanganate solution containing 2.5% of sodium chloride.* If a large amount of permanganate is required and the solution is dark brown, start afresh with a smaller aliquot. In case too much permanganate has been added (as indicated by a persistent deep color), add dropwise *3% hydrogen peroxide solution containing 1% of glacial acetic acid* until the color of the liquid other than the flocks of manganese peroxide is straw color or distinctly yellow. Heat 5 to 10 minutes on the water bath until the manganese peroxide deposits on the bottom or floats in part on the surface, then filter through asbestos and wash free of chlorine.

Color Formation. Concentrate the practically colorless filtrate in a porcelain dish on the water bath, transfer with a small amount of water to a 500-ml. volumetric flask, dilute to about 20 ml., and add 10 ml. of *10% sodium hydroxide solution* and 20 ml. of *saturated picric acid solution.* After 5 minutes, fill to the mark, mix, and filter if necessary.

Color Comparison. Determine the color value in the Duboscq colorimeter against a *0.5 N solution of potassium dichromate.*

Calculation. Obtain the milligrams of

creatinine by the formula: 8 ml. of 0.5 *N* $KMnO_4$ = 10 mg. of creatinine.

III. Vautier Modification.[105] Evaporate 0.25 to 3 g. of bouillon cubes with 5 ml. of *hydrochloric acid* and take up the residue in boiling water. Filter, add several drops of *hydrochloric acid,* and evaporate to dryness with stirring until sodium chloride crystallizes, then cool, add 10 ml. of water to the residue, and proceed as in the Folin-Benedict Method as given under Meat above.

IV. Verdino Photometric Modification.[106] By this modification (Graz University) the analysis may be completed within 2 hours.

APPARATUS. *Pulfrich Photometer,* with filter S 53.

REAGENT. *Picric Acid Solution,* cold, saturated. Dissolve 1.2 g. of the acid in 100 ml. of hot water, and cool. A mixture of 10 ml. of this solution and 1 ml. of 10% NaOH solution, made up to 25 ml., should show no absorption after at least 15 minutes, using filter S 53.

PROCESS. *Deproteinization.* Weigh 2 g. of the well-mixed sample into a beaker, add 20 to 30 ml. of water and 10 ml. of *0.667 N sulfuric acid,* and boil until all the soluble matter has dissolved. Wash thoroughly with hot water, transfer to a 100-ml. volumetric flask, add 10 ml. of *10% sodium tungstate solution,* make up to the mark, shake, and filter through a dry paper.

Acid Conversion. Pipet 5 ml. of the clear filtrate into a 100-ml. flask, add 1 ml. of *1.0 N hydrochloric acid,* heat at 130° for 20 minutes, in an autoclave, and cool 10 minutes.

Color Formation. Ignoring the usual turbidity, add 20 ml. of *cold saturated picric acid solution* and 1.5 ml. of *10% sodium hydroxide solution,* shake thoroughly, allow to stand 10 minutes, make up to the mark, and again shake.

Color Reading. Fill a 10- to 20-mm. cell (whichever gives a transmission of 20 to 50%) and read in the Pulfrich photometer against distilled water, using filter S 53.

CALCULATION. Obtain the milligrams of creatinine in 100 g. of the sample (C) by the formula:

$$C = E \times A \times 10^5 \times 10^3 = 2.6E \times 10^3$$

in which E is the extinction coefficient and A is the absorption coefficient ($= 2.6 \times 10^{-5}$). The calculation of E from the drum reading is facilitated by consulting the table in C. Urbach's book which gives the logarithm of the transmission. Multiply this log by -1 and divide by the cell length.

V. Woidich Modification.[107] The procedure for soup stock is essentially the same as in the Vautier modification except that the reading is made in an electric colorimeter and compared with a calibration curve.

VI. Remy Aluminum Hydroxide Modification.[108] Clarification is effected, without loss of creatinine, by a column of aluminum hydroxide, after which creatinine-picric acid is formed and compared with a standard.

APPARATUS. *Aluminum Hydroxide Cylinder,* 30 cm. high and 2.5 cm. in diameter. *Colorimeter.*

PROCESS. *Acid Conversion.* Weigh into a flask 20 g. of the finely divided meat or a corresponding amount of meat extract, add 150 ml. of *1.0 N hydrochloric acid,* and reflux for 1 hour. Transfer to a 200-ml. volumetric flask, neutralize with *10% sodium hydroxide solution,* make up to the mark, and filter through a dry pleated paper.

Clarification. Run the solution through a column of *aluminum hydroxide* 12 cm. high, thus obtaining a clear and colorless solution.

Color Formation. Pipet 10 ml. of the solution into a 200-ml. volumetric flask, add 12 ml. of a mixture of 4 ml. of *10% sodium hydroxide solution* and 8 ml. of *saturated picric acid solution,* allow to stand 10 minutes, and make up to the mark.

Color Comparison. Use for the comparison an aliquot of a stock solution of *10% pure creatinine* suitably diluted and treated in the same manner as the unknown.

Examples. Remy reports on samples of muscle on the fresh and dry basis as follows: horse 0.38 and 1.23, rabbit 0.49 and 1.93, beef 0.41 and 1.58, pork 0.33 and 0.89, and veal 0.36 and 1.11% of creatinine.

VII. Kácl and Fink Zinc Hydroxide Modification.[109] The originators of this modification believe that it should be adopted in preference to their trichloroacetic modification, as well as the original Sudendorf and Lahrmann modification and the Kácl and Fink photometric adaptation. Furthermore it is shorter than the trichloroacetic modification and is more accurate than all three procedures because the foreign "chromogenic substance" does not interfere to as great an extent. However, all the modifications studied tend to give high results.

Apparatus. *Pulfrich Photometer*, equipped with S 53 filter and 10-, 20-, and 30-mm. cells.

Autoclave.

Reagent. *Zinc Sulfate Solution*, 10%. From time to time dilute 10 ml. of the solution with 80 ml. of water and titrate with 0.5 *N* sodium hydroxide solution (10 to 11 ml.), using a few drops of 1% ethanolic phenolphthalein solution as indicator.

Process. *Clarification.* Weigh in duplicate into 100-ml. volumetric flasks 0.5 to 1.0 g. of a bouillon cube or 2 to 3 g. of a soup flavoring, and dissolve in 15 to 30 ml. of water. Dilute one of the solutions with 100 to 200 ml. of water, add *phenolphthalein solution*, and titrate with *standard 0.5 N sodium hydroxide solution.* To the other solution, add the number of milliliters of *standard alkali* necessary for neutralization as determined by the titration. To the neutralized solution add from a buret 10 ml. of the *10% zinc sulfate solution* whose titer had been determined, and mix, then add from a buret the number of milliliters of *0.5 N sodium hydroxide solution* corresponding to the titer of the zinc sulfate solution, and shake for 1 minute. After 10 minutes fill to the mark, shake for 1 minute, and filter through a dry pleated hardened paper, rejecting the first few milliliters of filtrate.

A. Creatinine. Pipet 5 to 10 ml. of the filtrate into a 25- to 100-ml. volumetric flask (depending on the content of preformed creatinine), add 10 ml. of *saturated picric acid solution* and 1 ml. of *10% sodium hydroxide solution,* and mix by swirling. After 8 minutes, make up to the mark and determine the percentage of transmission, using filter A 53 and a cell of suitable length (10, 20, or 30 mm.). Regulate the dilution and length of the cell so that the transmission is between 25 and 75%.

B. Total Creatinine. Treat 5 to 10 ml. of the filtrate with 1 to 2 ml. of *1.0 N hydrochloric acid,* heating either at 130° in an autoclave for 20 to 25 minutes or at 105° in a drying oven for 45 to 60 minutes. Cool, add 10 ml. of *saturated picric acid solution* and 1.5 ml. of *10% sodium hydroxide solution,* and proceed as under *A*.

Calculation. Obtain the milligrams per 100 g. of total creatinine (C) from the equation

$$C = 2.6 \times E \times Z$$

in which E is the extinction coefficient (derived from the table accompanying the Pulfrich photometer or supplied by Zeiss) and Z is the final dilution. If instead of a cell 10 mm. long, one 20 or 30 mm. is used, divide E by 2 or 3 respectively.

The equation is a simplication of the following:

$$C = A \times E \times 10^5 \times Z$$

and

$$A = 2.6 \times 10^{-5} \text{ (absorption coefficient)}$$

Substituting the value for A,

$$C = 2.6 \times 10^{-5} \times E \times 10^5 \times Z$$
$$= 2.6 \times E \times Z$$

Examples. On three samples of soup flavoring Kácl and Fink obtained results in terms of milligrams per 100 g. of creatinine as follows:

	Sudendorf and Lahrmann		Modified Sudendorf and Lahrmann		Direct Determination		Verdino		Trichloroacetic Acid		Zinc Hydroxide	
	Preformed	Total	Preformed	Total	Preformed	Total	Preformed	Total	Preformed	Total	Preformed	Total
I	415	425	422	428	468	474	362	372	372	372	263	269
II	850	850	849	849	866	879	826	826	838	838	785	785
VI	1001	1601	1023	1578	999	1659	976	1528	984	1552	976	1461

VIII. Kácl and Fink Trichloroacetic Acid Modification.[110] This modification differs from the zinc hydroxide modification in the following details: (1) the preliminary neutralization with 0.5 *N* sodium hydroxide is omitted; (2) 15 ml. of *20% trichloroacetic acid solution* is substituted for the zinc sulfate solution and the equivalent amount of 0.5 *N* sodium hydroxide solution; and (3) the autoclaving is continued for 40 minutes.

Purine Bases

Micko found that hypoxanthine is the chief purine of meat extract; xanthine and adenine are also present. Guanine, which is present in meat, appears to be destroyed or removed in the process of manufacture.

Structural formulas are given above under Meat.

Krüger–Micko Copper–Silver Gravimetric Method.[111] This method was employed by Micko in his classical studies of meat and yeast extracts and bouillon cubes.

Process. *Copper Precipitation.* Reflux 5 g. of the sample with 100 ml. of water and 10 ml. of *1 + 3 sulfuric acid* for 3 hours, cool, and neutralize with *sodium hydroxide solution.* To the mixture, add 20 ml. of *13% copper sulfate solution* and 20 ml. of *20% sodium bisulfite solution,* boil 2 to 3 minutes, and cool. Filter and wash with water containing a little of both reagents, thus avoiding oxidation. Transfer the precipitate and paper to a flask, add a little water and sufficient *hydrochloric acid* to dissolve the precipitate, then boil to disintegrate the paper and remove sulfur dioxide.

Copper Removal. Pass *hydrogen sulfide gas* into the flask to precipitate the copper, filter, after allowing to stand overnight, through a close-textured paper, and wash with water containing a little *hydrogen sulfide* and a few drops of *hydrochloric acid.* Evaporate the filtrate to small volume, but not to dryness, add a little hot water and a few drops of *hydrochloric acid,* nearly neutralize with *1 + 1 ammonium hydroxide,* then after diluting to 70 ml. and cooling, add 20 ml. more.

Silver Precipitation. To the ammoniacal liquid, add dropwise with stirring a mixture of 25 ml. of *10% silver nitrate solution* and 25 ml. of *1 + 1 ammonium hydroxide.* Allow to stand overnight in a dark place, filter, and wash three times with *1 + 6 ammonium hydroxide,* then with *1 + 40 ammonium hydroxide* until all nitrates are removed. Remove ammonium hydroxide by washing with *70% by volume ethanol,* dry in a water oven, and determine nitrogen.

Calculation. Express result as percentage of purine nitrogen or of purines calculated as xanthine by the factor 2.71.

Amino Acids

See also Part I, C4*b*.

Baur and Barshall Naphthalenesulfochloride Volumetric Method.[112]

Process. *Phosphotungstate Precipitation.*

Dilute 15 g. of the extract with 10 volumes of water, add a mixture of 125 ml. of *2 + 10 sodium phosphotungstate solution* and 45 ml. of *1 + 3 sulfuric acid,* and allow to stand at least 2 days. Filter, wash with *dilute sulfuric acid,* neutralize the filtrate with *sodium hydroxide,* and add a further quantity in the proportion of 0.4 g. for each 100 ml. of solution.

β-Naphthalenesulfochloride Precipitation. Remove the calcium phosphate by filtration and add to the filtrate 150 ml. of a *5% β-naphthalenesulfochloride solution in ether,* and shake in a suitable device for 18 hours. Separate the ethereal layer, supersaturate the aqueous portion with *hydrochloric acid,* and allow to stand 1 day.

Nitrogen Determination. Collect the precipitate on a filter of known nitrogen content, extract the filtrate if necessary with *ether* until clear, evaporate the ethereal extract, combine with the precipitate, and determine nitrogen in the whole by the Kjeldahl method. Correct the result for the amino nitrogen remaining in solution by adding for each 100 ml. of solution 0.002 g. of nitrogen.

Benedict and Murlin Method.[113] This method, devised for urine, may be adapted for meat extracts. The distinctive features are (1) removal of bases and ammonia by precipitation with *10% phosphotungstic acid* (Merck) in a *2% hydrochloric acid solution,* and (2) precipitation of the excess of phosphotungstic acid with *solid barium hydroxide.*

Glycerol

See also Part I, C9*c*, and Part II, F1.

Before the adoption of the U. S. Standards, glycerol was often added to meat extracts to prevent spoilage or to improve the consistency.

Hehner-Cook Acetone-Silver Nitrate Method.[114] Process. *Acetone Extraction.* To a flat-bottom lead dish containing 20 g. of *ignited quartz sand,* add 2 g. of the solid or 5 g. of the liquid extract, then transfer the contents to a mortar and mix with more *sand* and several grams of *anhydrous sodium sulfate.* Introduce the mixture into a Soxhlet extractor, provided with a loose plug of cotton serving as a filter, or a large-size Johnson extractor, and extract with *anhydrous acetone* for 10 hours.

Silver Precipitation. Remove the acetone by distillation and finally by exhaustion with a vacuum pump. Dissolve the glycerol in water, add 5 ml. of *10% silver nitrate solution,* make up to 100 ml., allow to stand overnight, and filter.

Oxidation with Dichromate. Oxidize an aliquot of the solution with 30 ml. of *concentrated potassium dichromate solution* and 24 ml. of *sulfuric acid* and proceed in other details as directed for the Hehner-Ross Oxidation Method (Part II, D3).

Yeast Gum

Micko Test.[115] Prepare a solution of 1 part of the material in 3 parts of hot water, add a moderate excess of *ammonium hydroxide,* allow the precipitate to settle, and filter. To the cooled filtrate, add an excess of a freshly prepared mixture of 100 ml. of *13% copper sulfate solution,* 150 ml. of *ammonium hydroxide,* and 300 ml. of *14% sodium hydroxide solution,* and shake violently.

In the presence of 10% of yeast extract in meat extract, a gray-white flocculent precipitate appears.

Sugars

Employ the methods described under Meat but weigh a quantity of the sample about one-tenth that directed for meat.

Ash

See Part I, C2*f* and 8*a* and *b*, for methods for the determination of ash and ash constituents.

Common Salt

See Meat above.

4. ANIMAL FATS AND OILS

See Part II, B2.

5. FISH AND SHELLFISH

Drip

Taylor [116] has shown that the drip (loss on defrosting) of fish fillets, which is practically nil before freezing, gradually increases after either quick or slow freezing to a maximum in 1 to 2 months. Eventually the drip diminishes owing, in large part, to the movement of moisture-laden air currents toward the freezing units.

Taylor Indirect Gravimetric Method. The weighed fillet is suspended over a funnel which delivers the drip into a measuring cylinder. The percentage of drip, however, is calculated from the loss in weight during defrosting and not from the weight of the drip which is less owing to evaporation. By conducting the operation in a cabinet, the loss by evaporation is reduced. Nitrogen is determined in the drip.

Winton Direct Technique. The writers prevent practically all loss through evaporation by suspending the fillet from a metal cover in a percolator and collecting the drip in a closely fitting, but not air-tight, tared salt-mouth bottle (Fig. 179). Use weight of the drip for the calculation of the percentage, but check the result by calculation from the loss in weight of the fillet.

Make the total drip up to the mark in a volumetric flask and use aliquots for the determination of solids, nitrogen, ash, and ash constituents.

If desired cut the fillet longitudinally into approximate halves and use one half for the determination of drip and the other half, after being ground in a meat chopper while still frozen, for the determination of moisture, protein, fat, and ash, assuming that the two halves are of the same composition.

Examples. The drip of frozen fillets varies greatly with the species, time of storage, and other conditions. The authors named found that in extreme cases the drip of haddock

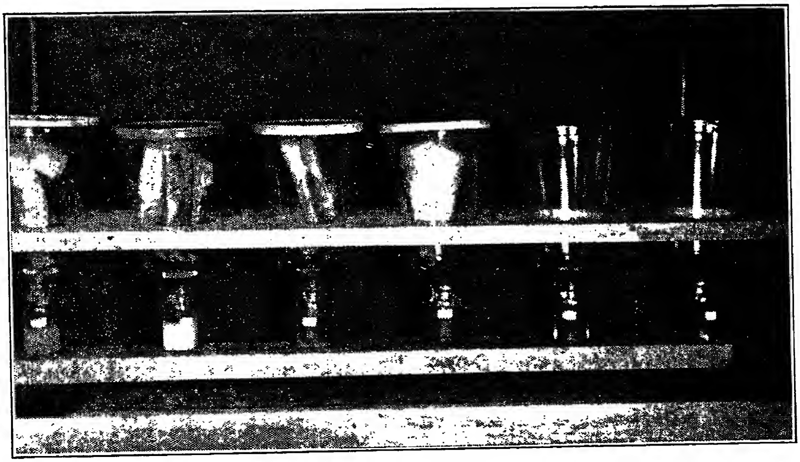


Fig. 179. Multiple Fish-Fillet Drip Apparatus.

fillets may reach 25% and the protein (largely coagulable) of the drip may amount to 5% of the total protein in the fillet.

NOTE. Experiments on the use of separate charges of 10 g. each of the ground frozen fish for the determination of drip, as well as chemical constituents, were interrupted by war restrictions.

MICROSCOPIC EXAMINATION

Cut transverse sections of the frozen fillets with a Gillette blade in a room cooled below the freezing point. Lay a cover glass on each section without crushing and examine at a magnification of 160 diameters. The sections of both quick and slow frozen fillets show small pockets between the muscle fibers, those of the former being smaller and more numerous than those of the latter.

VOLATILE NITROGENOUS CONSTITUENTS

(Trimethylamine, Ammonia, Primary and Secondary Amines)

Okoloff Magnesium Oxide Distillation Volumetric Method.[117] Although not employing any strikingly novel features, the method represents a careful study of reactions with practical application in the detection of spoilage in herring, as well as other species of fish, and probably other meat products.

PROCESS. *Extraction.* Remove entrails and bones, run through a meat grinder, and soak 50 g. of the ground sample with 1 liter of water for 24 hours in a refrigerator. On the following day, shake, allow to stand 30 minutes, and filter through cheese cloth.

A. TOTAL VOLATILE NITROGEN. *Distillation.* Measure 400 ml. of the filtrate into each of two distillation flasks, add 30 ml. of *ethanol* and 2 g. of *magnesium oxide* to each flask, attach each to an inclined condenser, and distil into a 1-liter Erlenmeyer flask containing 25 ml. of *0.1 N sulfuric acid*, continuing the distillation until about three-fourths of the liquid has passed over, the exact stopping point being determined by testing with *litmus paper*. Boil both distillates until the carbon dioxide has been removed (10 to 15 minutes).

Titration. To both distillates add 0.2 ml. of *0.2% rosolic acid indicator in 80% ethanol* and titrate the excess of acid with *0.1 N alkali*.

CALCULATION. Obtain the milligrams of total volatile nitrogen per 100 g. of fish by multiplying the number of milliliters of bound acid by 7.0 ($= 1.4 \times 5$).

Slightly acidulate one of the titrated distillates, cool under the tap, stopper, and store in the refrigerator for the determination of ammonia, C below.

B. TRIMETHYLAMINE NITROGEN. Transfer the second titrated distillate from the receiver to a distillation flask, rinse, and make up to about 300 ml. Add according to the amount of volatile acids 15 to 20 g. of *sodium nitrite* and 25 ml. of *acetic acid*. Heat the mixture for 40 to 60 minutes over a small flame under the outlet; if crystals form on the wall of the flask, lower the flame. Dilute the highly concentrated liquid to the former volume, then add *pumice*, attach a piece of *litmus paper* to the wall, and add 50 ml. of *30% sodium hydroxide*. Attach to the condenser, shake so as to wet the litmus paper (which must be colored blue), and distil into a receiver containing 25 ml. of *0.1 N acid* until three-fourths of the liquid has distilled over. Distil as before over a small flame, lowering the flame if crystals form on the wall. Determine the end of distillation by *litmus paper*. Boil the distillate 15 minutes to remove carbon dioxide, then cool and titrate the excess of acid with *0.1 N sodium hydroxide* solution, using *rosolic acid indicator*.

CALCULATION. Obtain the milligrams of trimethylamine nitrogen per 100 g. of fish by

multiplying the number of milliliters of bound 0.1 *N* acid by 7.0 (= 1.4 × 5).

C. Ammonia Nitrogen. To the titrated distillate obtained under A which was stored in the refrigerator, add *0.1 N alkali* to exact neutrality (faint pink), followed by 20 ml. of clear *formaldehyde solution* previously titrated with *0.1 N alkali* to a distinct rose color, then titrate the whole with *standard 0.2 N alkali*.

Calculation. From the total number of milliliters of 0.2 *N* alkali employed in the titration, subtract the number required for the neutralization of the formaldehyde, then multiply the difference by 14.0 (= 2.8 × 5), thus obtaining the milligrams of ammonia nitrogen per 100 g.

D. Other Amine Nitrogen. If the sum of B and C is less than A, then A − (B + C) represents approximately the nitrogen of primary and secondary amines.

Examples. The following results in milligrams per 100 g. by Okaloff illustrate the application of the method:

	Ocean		Astrakan		Kertsch	
	Sound	Spoiled	Sound	Spoiled	Sound	Spoiled
Total volatile *N*	40 to 90	54 to 185	20 to 40	40 to 41	15 to 30	80
Trimethylamine *N*	28 to 65	43 to 182	16 to 33	27 to 33	12 to 29	70
Ammonia *N*	4 to 20	....	0.9 to 5	3 to 7	0 to 3	..

Volatile Basic Nitrogen

Lücke and Geidel Magnesium Oxide Distillation Volumetric Method.[118] The procedure (Wesermünde Fisheries Institute) is an adaptation of the usual magnesium oxide method for the determination of ammonia nitrogen.

Process. *Distillation.* Place 5 to 10 g. of the comminuted sample in a flask and add 300 ml. of water, 1 to 2 g. of *magnesium oxide*, and a few drops of *octyl alcohol*, or a few milliliters of *paraffin oil*, to prevent foaming. Heat to boiling (10 minutes) and distil for 25 minutes, collecting the distillate in *0.1 N sulfuric acid.*

Titration. Back-titrate with *standard 0.1 N sodium hydroxide solution.*

Calculation. Express results in milligrams of volatile basic nitrogen per 100 g.

Examples. A limit of about 20 mg. of volatile basic nitrogen per 100 g. characterizes fresh fish. The amount may rise to 30 mg. without serious detriment, but when over 30 mg. the keeping quality is lessened and at 50 mg. the fish is spoiled.

Ammonia

See Meat, above.

Note. Kimura and Kumakura[119] state that fresh fish muscle shows less than 10 mg. of ammonia and amine nitrogen per 100 g. If above 20 mg. decomposition has begun, and if above 30 mg. the decomposition is well advanced.

Glycogen

Pflüger Alkali-Ethanol Copper Reduction Method. See Meat above.

Calderwood and Armstrong Modification.[120] Although applied by Calderwood and Armstrong to oysters, the procedure appears suit-

able for other tissues. It was developed under the auspices of the U. S. Fish and Wildlife Service, the Virginia Commission of Fisheries, and The College of William and Mary.

PROCESS. *Digestion.* Obtain by difference the weight of the charge (5 to 10 g.) calculated to yield not over 0.4 g. of glycogen. To the charge in a 400-ml. beaker add 10 to 20 ml. (2 ml. per g.) of *30% carbonate-free 3 + 7 sodium hydroxide solution*, cover, and digest on the steam bath at 80° (±10°) with occasional stirring for 30 minutes.

Ethanol Precipitation. Disintegrate any lumps with a mushroomed stirring rod, add 100 ml. of water, mix thoroughly, add 135 ml. of *ethanol*, and stir only enough to mix thoroughly. Cover, let settle at room temperature overnight. Decant the supernatant liquid onto an 18-cm. Whatman No. 54 filter paper folded at 90°, in a fluted funnel. Wash four times by decantation with 75-ml. portions of *66% (2 + 1) ethanol*, then transfer the precipitate to the filter and wash twice with the *ethanol*. The precipitate will be white or gray according as mud is absent or present.

Hydrolysis. Close the end of the funnel with a piece of rubber tubing and a clamp, fill with hot water, cover, and let stand 2 or 3 hours or overnight. Open the clamp and allow the solution to drain into a 250-ml. volumetric flask. Attach the clamp and fill with hot water as before, but allow to stand only 1 hour, then drain. Repeat the treatment until the filtrate, which at first is turbid, is clear, limiting the total solution, however, to 175 ml. Add 12.5 ml. of *hydrochloric acid*, mix, and heat in a boiling water bath for at least 4 hours, taking care that the solution is kept above 92°.

Copper Reduction. Cool, neutralize to *phenolphthalein* with *20% sodium hydroxide solution*, again cool, dilute in a volumetric flask, and determine the dextrose by copper reduction by one of the methods described in Part I, C6*a*.

CALCULATION. Weight of dextrose × 0.9 = weight of glycogen.

SPOILAGE VALUES

Stansby and Lemon Electrometric Method.[121] The following procedure, developed at the U. S. Bureau of Fisheries, Gloucester, Mass., depends on the disappearance of the protein molecule instead of the formation of any one end product.

APPARATUS. *Platinum Electrode.*

Calomel Half-Cell.

Potentiometer.

PROCESS. *Extraction.* Grind 20 g. or more of the bone-free fish as fine as possible in a grinder. Weigh 5 g. into a 150-ml. bottle, add 70 ml. of water, and shake for at least 10 minutes in a shaking apparatus. Transfer the contents of the bottle to a 200-ml. Erlenmeyer flask and add an excess (about 0.3 g.) of *quinhydrone*, rinsing the bottle with 30 ml. of water added to the contents of the flask. Shake for an additional 2 minutes and transfer to a 250-ml. beaker.

Potentiometric Reading. Insert a platinum electrode and saturated calomel half-cell, then connect with a potentiometer. Take readings until a "constant" value is obtained, then add successive portions of *0.0165 N hydrochloric acid* from a buret, stirring the solution and waiting for equilibrium between each addition. Avoid too rapid addition, especially between $E = 0.140$ and $E = 0.170$ since otherwise a drifting potential is obtained near $E = 0.200$. Obtain several potentiometer readings between $E = 0.200$ and $E = 0.100$, the entire titration requiring between 15 and 30 minutes.

CALCULATION. Obtain the amount of acid used to reach $E = 0.100$ ($p\text{H} = 5.97$), as well as the amount used to go from $E = 0.100$ to $E = 0.200$ ($p\text{H} = 4.28$).

The value B is taken as the number of milliliters of 0.0165 N hydrochloric acid re-

quired to bring the pH of 5 g. of fish in 100 ml. of water to 6.0. More exactly, since these pH values ignore protein error, the value of B is the number of milliliters of 0.0165 N hydrochloric acid required to bring the electromotive force to $E = 0.100$ volt, which is assumed to be proportional to the amount of secondary decomposition.

The value of A is the number of milliliters of 0.0165 N hydrochloric acid required to change E from 0.100 to 0.200 volt, which corresponds to a pH change from about 6 to 4.3 and is inversely proportional to the amount of primary changes.

EXAMPLES. The following values of B and the correlated results of organoleptic examination made up to 18 days after catching are illustrative of the application of the method:

B 4 to 6: odor fresh, secondary change none
B 6 to 8: odor fresh or slightly fishy, secondary change none or slight
B 8 to 10: odor fishy or slightly fishy, secondary change slight
B 10 to 12: odor fishy or stale, secondary change small or pronounced
B 12 to 14: odor stale or very stale, secondary change pronounced or considerable
B 14 to 17: odor very stale or putrid, secondary change considerable or extreme
B 17 plus: odor putrid, secondary change extreme

PRESERVATIVES

See Part I, C13.

6. FISH OILS AND FISH LIVER OILS

See Part II, B2.

7. EGGS

Composition of Hens' Eggs.[122] Beythien the following composition of the dry edible portion of eggs, egg white, and egg yolk, containing respectively 73.67, 50.93, and 81.61% of water:

	Whole egg	Yolk	White
Protein	47.81	33.12	88.79
Fat	45.99	64.10	1.76
Cholesterol	1.22	1.92	....
Lecithin	11.22	18.97	....
Lecithin P_2O_5	1.06	1.67	....
Total P_2O_5	1.58	2.72	0.22
NaCl free ash	4.25	2.08	4.21

Analytical Methods. Analyses of fresh whole eggs serve in calculating the nutritive value and as standards in following the changes during ordinary and cold storage and in judging the purity and freshness of frozen opened eggs and dried eggs. Lecithin and so-called lecithin phosphoric acid (phosphoric acid soluble in ethanol or other solvents) are the chief characteristic constituents. The determination of ammonia and acidity of the fat (see Meat) serve in detecting deterioration.

The methods which follow have been largely developed in the laboratories of the U. S. Food Administration.

MOISTURE

See Meat above. In the Official A.O.A.C. Method, the drying is conducted at 98 to 100° *in vacuo* (25 mm.). Lepper[123] found that lower results are obtained by drying *in vacuo* at 55°.

TOTAL NITROGEN

See Part I, C1*c*.

WATER-SOLUBLE NITROGEN; CRUDE ALBUMIN NITROGEN

Alfend-Mitchell Modification of the Gravimetric Method.[124] PROCESS. A. LIQUID

Eggs (Official). Weigh 10 g. of the well-mixed sample into a 250-ml. volumetric flask containing 150 ml. of water, add 5 ml. of *0.01 N acetic acid* for each gram of egg substance, dilute to volume, shake gently, and filter through a dry paper, avoiding evaporation and returning the first portions to the paper if cloudy.

B. Dried Eggs (Tentative). Weigh 1 g. of egg white, 3 g. of whole eggs, or 5 g. of egg yolk into a 250-ml. nursing bottle, add 50 ml. of *naphtha*, mix gently, centrifuge, and decant the solution. Repeat the treatment once. Place the bottle on its side and roll occasionally until the residue is dry, then break up the lumps. Add 100 ml. of water, at first slowly with gentle mixing until the material disintegrates, then add 5 ml. of *0.01 N acetic acid* for each gram of egg substance present, transfer with water to a 200-ml. volumetric flask, and dilute to volume. Mix gently, let stand 2 hours with occasional shaking, centrifuge, and filter through dry paper.

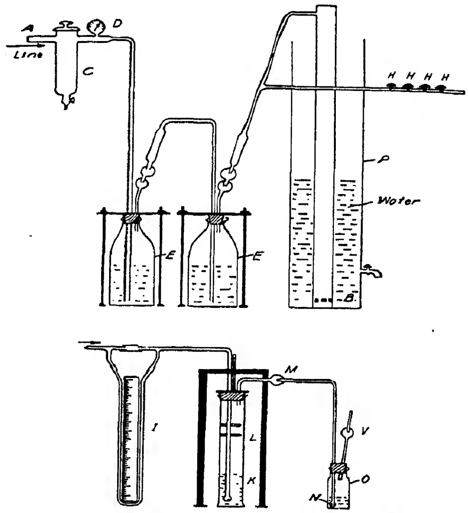
Water-Soluble Nitrogen. Determine nitrogen by the Kjeldahl Method (Part I, C2*b*) in 50 ml. of the clear filtrate.

Crude Albumin Nitrogen. Pipet 100 ml. of the clear filtrate into a 200-ml. flask, add 15 ml. of *sodium chloride solution* (28 g. diluted to 300 ml.), fill nearly to the mark with *ethanol*, mix, cool to room temperature, fill to the mark, and let stand overnight. Filter through a dry paper, determine the percentage of nitrogen by the Kjeldahl method in 100 ml. of the filtrate, and subtract from the percentage of water-soluble nitrogen to obtain the percentage of crude albumin nitrogen.

Ammonia Nitrogen

Thomas and Van Hauwaert Potassium Carbonate Aeration Volumetric Method.[125] The method is based on the results of studies made at Columbia University.

Apparatus. *Aeration Train* (Fig. 180). *A* air pipe connecting aeration apparatus with air line, *C* air filter, *D* reducing valve permitting reduction of line air pressure, *E* wash bottles containing 35% sulfuric acid to remove ammonia from entering air, *H* valves for further adjustment of air flow, *I* flow meters, *K* aeration cylinder, *L* rubber baffles, *M* spray trap, *N* dispersion tube with perforated bulb, *O* receiver containing standard acid, *P* pressure regulator, and *V* outlet tube with spray trap.



Courtesy of Ind. Eng. Chem., Anal. Ed. 1934, 6, 339

Fig. 180. Thomas and Van Hauwaert Aeration Train.

Process. *Charge.* Measure exactly 10 ml. of *standard 0.02 N sulfuric acid* into *O* and dilute with 50 ml. of water. Place 1 g. of *sodium fluoride* and 25 g. of the mixed egg magma, weighed by difference from a weighing bottle, in *K*, then add 55 ml. of water, 50 ml. of *ethanol*, and 1 ml. of *kerosene*.

Aeration. Connect with *O*. Add 20 ml. of *5% anhydrous potassium carbonate solution*, close the aeration cylinder, and start the aeration, allowing 1200 liters of air to pass at the rate of 240 liters per hour.

Titration. By means of a micro buret reading to 0.01 ml., titrate the excess of acid with *standard 0.02 N sodium hydroxide solution* (carbon dioxide-free).

Blank. Run simultaneously two blank determinations with 1 g. of *sodium fluoride*, 80 ml. of water, 50 ml. of *ethanol*, 1 ml. of *kerosene*, and 20 ml. of *50% potassium carbonate solution*.

Calculation. Deduct from the number of milliliters of standard alkali used in the blank determination that used in the actual analysis and multiply the difference by 1.12 to obtain the milligrams of nitrogen as ammonia per 100 g. of sample.

Note. The Tentative A.O.A.C. Method differs from the above chiefly in that the sample and fluoride are diluted with four 25-ml. portions of water and 75 ml. of ethanol and in that the time required for aeration is determined by a test employing 20 ml. of NH_4Cl or $(NH_4)_2SO_4$ solution about 5% stronger than 0.02 *N*.

"Lipoids"

The continued use of the terms lipoids and lipoid phosphoric acid, which have been dropped by McLean, Mathews, Gortner, Bodansky, and others, is confusing. The term ethanol-chloroform extract and Juckenack's term lecithin-phosphoric acid seem less open to criticism.

Hertwig Ethanol-Chloroform Method.[126] Reagent. *Mixed Solvent.* Equal parts of chloroform and absolute ethanol.

Process. A. Liquid Eggs. To 4 g. of the sample in a 100-ml. volumetric flask, add dropwise from a pipet 25 ml. of the *mixed solvent*, shaking continually until the proteins coagulate and the coagulum breaks up. Add 60 to 65 ml. more of the *mixed solvent* and allow to stand 1 hour, shaking at 5-minute intervals. Fill to the mark with the *solvent mixture*, shake, and let stand until clear.

B. Dried Eggs. Treat 2 ml. of the sample in a 100-ml. volumetric flask with 85 to 90 ml. of the *mixed solvent*, let stand 1 hour, shaking at 5-minute intervals, and proceed as above.

Lipoids Extraction. Evaporate to dryness on the steam bath 50 ml. of the solution of the sample in a 150-ml. beaker and dry at 100° for 5 to 10 minutes. Dissolve the dried residue in 5 to 10 ml. of *chloroform*, filter into a weighed 100-ml. Pyrex beaker through a pledget of cotton, and rinse with *chloroform*. Evaporate on the steam bath and dry at 100° to constant weight (90 minutes), allowing the beaker to stand in the air for 30 minutes before weighing.

Calculate the percentage of "lipoids" (ethanol-chloroform extract).

"Lipoid Phosphoric Acid"

Saponification Method.[127] Reagent. *Ethanolic Sodium Hydroxide Solution.* Dissolve 100 g. of carbonate-free NaOH in 100 ml. of water, allow to stand until clear, and filter through a hardened paper soaked in ethanol. Mix 50 ml. of this solution with 900 ml. of ethanol and dilute to 1 liter with the same solvent.

Process. Dissolve the dried "lipoids," obtained in the determination of "lipoids" above, in 2 to 3 ml. of *chloroform*, add 10 to 20 ml. of *ethanolic sodium hydroxide solution*, and evaporate to dryness on the steam bath, avoiding spattering, then dry at 100° for 30 minutes. Incinerate for 1 hour in a muffle furnace at 500°, cool, stir the residue with a few drops of water, and take up in 5 ml. of *1 + 3 nitric acid.* Filter, wash, and determine phosphoric acid in the filtrate (Part I, C8*a*).

Report as lipoid phosphoric acid.

Fat

Hertwig Modification of Schmidt-Werner Acid Hydrolysis Method.[128] Process. A.

LIQUID EGGS. Weigh into a Röhrig or Mojonnier fat extraction tube about 2 g. of yolk, 3 g. of whole egg, or 5 g. of white. Add slowly with shaking 10 ml. of *hydrochloric acid*, place in a water bath at 70°, and heat to boiling, continuing the boiling for 30 minutes with shaking at 5-minute intervals. Remove from the bath, nearly fill to the lower bulb with water, and cool to room temperature.

B. DRIED EGGS. Place 1 g. of the sample in the fat extraction tube, add slowly, with washing down the sides, 10 ml. of *4 + 1 hydrochloric acid*, then proceed as above.

Fat Determination. To the extraction tube, add 25 ml. of *ether* and mix, then add 25 ml. of redistilled *naphtha* (m.p. below 60°), let stand until the solvent layer is clear, and proceed as directed for the Hertwig Acid Hydrolysis Method (Part II, A2), but omitting the filtration.

ACIDITY OF FAT

Two methods (the first official, the second tentative) involving titration with sodium ethylate have been developed by members of the Association of Official Agricultural Chemists.[129]

I. Dry Ether Extraction Volumetric Method [130] (Not suited for egg white). REAGENTS. *Benzene.* Best available quality; if not neutral, titrate 50 ml. with 0.05 *N* sodium ethylate and correct results accordingly.

Standard Sodium Ethylate Solution, 0.05 *N*. Dissolve about 1 ml. in volume of metallic sodium in 800 volumes of absolute ethanol. Titrate 10 ml. of 0.1 *N* HCl with the solution and adjust accordingly on the day the solution is to be used.

PROCESS. A. DRIED EGGS. *Desiccation.* Dry to constant weight 2 g. of the powdered sample in a tared 63-mm. aluminum dish at 55° under pressure not exceeding 125 mm. of mercury.

Ether Extraction. Transfer the residue to a 12.5-cm. hardened filter paper folded once, then place on a 15-cm. qualitative paper, roll into a cylinder, extract for 3 hours with *absolute ether* in a Knorr or other continuous extractor, and weigh. Evaporate the ether and dry the extract 1 hour at 55° *in vacuo* as before.

Titration. Dissolve the residue in 50 ml. of *benzene* and titrate with *standard 0.05 N sodium ethylate solution,* using 3 or 4 drops of *phenolphthalein indicator.*

CALCULATION. Report as milliliters of 0.05 *N* sodium ethylate solution per gram of ether extract.

B. LIQUID EGGS. Dry 5 g. of the sample to constant weight in a tared lead dish. Place the dish on a 12.5-cm. hardened filter paper, cut the sides of the dish, place another paper on top, and roll into a cylinder, then extract and titrate as above.

II. Wet Ether–Naphtha Extraction Rapid Volumetric Method [131] (For Liquid Eggs). REAGENT. *Ethanolic Brine Solution.* Dissolve 10 g. of sodium chloride in 50 ml. of water, add 30 ml. of ethanol, and dilute to 100 ml. with water.

PROCESS. *Extraction.* Weigh 10 g. of mixed whole liquid eggs or 5 g. of liquid yolk, mix well, wash into a centrifuge bottle with 40 ml. of *ethanolic brine solution*, and shake gently but thoroughly. Add 50 ml. each of *ether* and *naphtha* and repeat the shaking. Centrifuge and remove the yellow solvent layer. If only acidity of the ether extract is desired, two extractions, the second with 30 ml. each of the *solvents*, are sufficient.

Evaporation. Remove the solvents on the steam bath, add 5 ml. of *absolute ethanol*, and again evaporate. Dissolve the residue in *chloroform,* filter into a tared beaker, evaporate, and weigh.

Titration. Proceed as under I.

UNSAPONIFIABLE MATTER

Direct Saponification Gravimetric Method. The following revised method employs fea-

tures proposed by Kerr and Sorber,[132] Hertwig, Jamieson, Baughman, and Bailey[133] and Haenni.[134] The unsaponifiable matter is extracted with ether, not naphtha ("petroleum benzin") as in the method of the Fat Analysis Committee of the American Chemical Society.

PROCESS. *Saponification.* Weigh into a 125-ml. Erlenmeyer flask about 2.5 g. of whole egg, 1.5 g. of yolk, 1 g. of dried whole egg, or 0.7 g. of dried yolk and add 10 ml. of *6 + 4 potassium hydroxide solution.* Cover, heat for 3 hours on a steam bath with occasional swirling, cool until just warm, add 30 ml. of *ethanol*, and disperse any solid matter by swirling.

Ether Extraction. Mix by swirling with 50 ml. of *ether*, transfer to a 500-ml. separatory funnel, rinse the flask with two more 50-ml. portions of *ether*, and again mix by swirling. Rinse the flask with 100 ml. of *1% potassium hydroxide solution* while swirling the liquid in the funnel, continuing 10 to 15 seconds thereafter.

Soap Solution Removal. After allowing to stand 10 minutes, slowly draw off the soap solution up to the emulsion into a 250-ml. separatory funnel, rinsing with 10 ml. of *1% potassium hydroxide solution*, then add 50 ml. of *ether*, stopper, shake well, let separate, and draw off the aqueous liquid. Transfer the ether solution to the 500-ml. funnel, rinsing with 10 ml. of *ether*, and wash as before with 100 ml. of *1% potassium hydroxide solution.* Add 20 ml. of *1 + 4 hydrochloric acid*, swirl, then add 100 ml. of water and swirl again. Wash the ether solution twice more with 100 ml. of *1% potassium hydroxide solution*, testing the last washings for soap with *1 + 4 hydrochloric acid*, which should not produce a turbidity, then wash by swirling successively, with 50 ml. of water, 50 ml. of water containing 0.5 ml. of *0.1 N hydrochloric acid*, and twice with 50 ml. of water.

Desiccation, Filtration, and Evaporation. Filter the ether solution into a dry 300-ml. Erlenmeyer flask through 15 g. of *anhydrous sodium sulfate* in a sintered-glass filter, carefully regulating the suction. Rinse and wash with 10, 5, 5, and 5 ml. of *ether* into an Erlenmeyer flask containing a porcelain chip. Evaporate the ether on a steam bath, dissolve the residue in 20 ml. of dried *ether*, and transfer with 10-, 5-, and 5-ml. portions of dried *ether* to a tared 50-ml. Erlenmeyer flask containing a porcelain chip. Evaporate the ether, dry at 100 to 105° for 1 hour, and weigh.

CALCULATION. Deduct the weight obtained in a blank determination.

CHOLESTEROL

Haenni Ether-Bromine Volumetric Method.[135] From the solution of the unsaponifiable matter above, cholesterol is precipitated as dibromide by a solution of bromine in carbon tetrachloride. The dibromide is oxidized to bromate by sodium hypochlorite, and the bromate is determined iodometrically, after removal of the excess of hypochlorite and addition of potassium iodide and hydrochloric acid, by titration with thiosulfate by the method of van der Meulen[136] as modified by D'Ans and Höfer[137] and Kolthoff and Yutzy.[138]

REAGENTS. *Bromine Reagent.* Weigh to 0.1 g. a narrow-mouth glass-stoppered 25-ml. flask containing 5 ml. of CCl_4, add 4 to 5 g. of bromine, and weigh again, then dilute with the solvent to a final concentration of 0.22 (±0.02) g. of bromine per milliliter. Prepare fresh every other day.

Methyl Red Indicator, 0.5% in 48% ethanol.

Sodium Hypochlorite Solution, about 1.0 *N*. Dissolve 88 g. of NaOH in 200 ml. of water, add 1500 ml. of crushed ice, and pass in chlorine until 71 g. are absorbed. Dilute to 2 liters and store in dark bottles in the refrigerator. Reject if less than 0.95 *N*.

Standard Sodium Thiosulfate Solution, 0.02

N. Prepare with CO_2-free water containing 1% of amyl alcohol.

PROCESS. *Bromine Precipitation.* Pack in ice (1) a 25-ml. graduated cylinder, (2) the *bromine reagent*, and (3) a filter tube with stirring rod. Cool the *80% acetic acid* to about $-5°$ in an ice-salt mixture.

Into the 50-ml. Erlenmeyer flask containing the unsaponifiable matter from the method above, pipet while rotating 2 ml. of *absolute ether*, stopper with a cork, swirl to liberate the porcelain chip, and pack in the ice bath to near the top. After not less than 10 minutes, add 0.20 ml. of the cold *bromine reagent*, swirl, stopper, and place in the ice bath. After 10 minutes, add rapidly from the cold cylinder 15 ml. of the cold *80% acetic acid solution*, stir for 3 minutes in the ice bath, and cool further for 10 minutes. Filter rapidly by suction on the filter tube into a receiving flask, rinse with 5 ml. of the cold *80% acetic acid solution* twice, cooling in the ice bath after each, then three times with cold water. Reject the filtrate and washings.

Bromate Solution. Dissolve the precipitate on the filter tube in 10 ml. of *ethanol* into a 300-ml. flask with the aid of suction and wash the test tube and filter once with 10 ml. and twice with 5 ml. of *ether*, and finally with 10 ml. of *ethanol*, gently stirring sand with each portion and allowing to stand for a minute before applying suction.

Alkali Treatment. Wash the stem with a few milliliters of *ether*, add 1 ml. of *1 + 1 potassium hydroxide solution* to the ether solution, mix, wash down the sides of the flask with 5 ml. of *ether*, and evaporate the ether and ethanol on the steam bath, finally removing the last traces of ethanol vapor with air.

Dibromide Oxidation to Bromate (Modified van der Meulen Method). To the alkaline residual liquid, add 40 ml. of hot water, mix, neutralize with *6 N hydrochloric acid*, using 1 drop of *methyl red indicator*, then add 10 g. of *sodium chloride*, 3 g. of *monosodium phosphate*, and 20 ml. of *1.0 N sodium hypochlorite solution*. Heat to boiling, remove the heat, and add immediately with care 5 ml. of *50% sodium formate solution*, then cool and dilute to about 150 ml. with water.

Titration. Add 5 ml. of *20% potassium iodide solution*, 1 to 2 drops of *5% ammonium molybdate solution*, and 25 ml. of *6 N hydrochloric acid*, then titrate rapidly with *standard 0.02 N thiosulfate solution*, using *1% starch solution as indicator*.

Blank. Introduce a correction as determined by a blank analysis starting with Alkali Treatment above.

Calculation. Obtain the milligrams of cholesterol (C) by the following formula:

$$C = 0.55 + 0.688 \times T$$

in which T is the corrected milliliters of 0.02 *N* thiosulfate solution required for the titration.

SUGARS

Mitchell Copper Reduction Gravimetric Method.[139] The distinctive feature is the removal of interfering substances in the preparation of the solution briefly as follows.

PROCESS. *Lime-Salt-Ethanol Treatment.* Weigh 25 g. of liquid eggs, 2.5 g. of dried egg whites, or 10 g. of dried yolks or whole eggs into a 250-ml. volumetric flask containing 1 g. of *calcium carbonate* and 50 ml. of *5% sodium chloride solution*. Add to the liquid egg immediately 130 ml. of *ethanol* with mixing; allow the dried egg product to stand for 1 hour with mixing every 5 minutes, then add 130 ml. of *ethanol.* Allow to stand long enough for the gas bubbles to rise, cool to room temperature, fill to volume, shake, and filter through a pleated paper. Pipet 150 ml. of the filtrate into a beaker, evaporate to 20 to 30 ml., cool, and transfer with water to a 100-ml. volumetric flask, holding the volume to 80 to 90 ml.

Phosphotungstic Acid Treatment. Add the dry *powdered acid* in small portions in slight

excess, mix, let stand until the bubbles rise, dilute to volume, shake, and filter. Remove the excess of phosphotungstic acid with very small portions of dry *powdered potassium chloride* and filter.

If a considerable amount of sugars is present, prepare another solution, using double the amounts of the *sodium chloride solution* and *ethanol* and make up to and pipet double the volumes.

Copper Reduction. Determine by the Munson and Walker Method before and after inversion (see Part I, C6*a*) and calculate the dextrose and sucrose.

If the double dilution was practiced, subtract the result for the 250-ml. dilution from that for the 500-ml. dilution.

Glycerol

Alfend Modification of the Hehner-Ross Method.[140] Alfend describes a qualitative fuchsin-sulfite method and an adaptation of the Hehner-Ross quantitative method which is not applicable in the presence of sugars (see Part II, D3). The first has been adopted as a Tentative Method, the second as an Official Method.

Mineral Constituents

In the *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists* are given detailed instructions for the incineration with *sodium carbonate* and preparation of the solution in *dilute nitric acid* preliminary to the determination of phosphoric acid (P_2O_5) and chlorine in liquid and dried eggs. The molybdic volumetric method and the silver nitrate-thiocyanate method respectively are used for the determinations. The procedures in both cases are official. Other mineral constituents, after burning to a white or gray ash, may be determined by the usual methods. See Part I, C8*a*.

Iodine

See also Part I, C8*b*.

Almquist and Givens Bromine Iodide Colorimetric Method.[141] Eggs are a convenient source of iodine in dietaries and may be of value in the cure or prevention of disease. Through addition of iodine compounds to poultry feeds, the element finds its way into the egg. For its determination, Almquist and Givens (California Agricultural Experiment Station, Berkeley) propose the following procedure.

Apparatus. *Hydrogen Ion Colorimeter*, Bausch & Lomb 3650–1, provided with a 75-watt frosted lamp in a Bausch & Lomb colorimeter lamp 2414.

Process. *Evaporation and Incineration.* Place the liquid contents of a number of eggs in a flask, add an equal volume of *ethanol* and 10 g. of *potassium hydroxide* per egg, then reflux for 16 to 24 hours. Place an amount of the brown liquid equivalent to one egg in a 500-ml. nickel crucible, evaporate to dryness on a hot plate, and incinerate in a muffle furnace for 4 hours at about 600°.

Extraction. Take up the ash in 50 ml. of hot water, filter, and wash the residue with hot water. Acidify carefully with *6 N sulfuric acid* to *methyl red* and add 5 more drops of *acid*. Add next *saturated bromine water* to a permanent strong yellow color, boil off the excess, evaporate to about 15 ml. on a steam bath, cool, and transfer to a small separatory funnel. Remove and wash any crystalline matter formed during the cooling and add the washings to the solution.

Potassium Iodide Treatment. Add a crystal of *potassium iodide* and extract the iodine formed with five 1-ml. portions of *carbon tetrachloride*.

Color Reading. Compare in the colorimeter with *standard solutions of iodine* in *carbon tetrachloride* in the colorimeter, using a 5-ml. cell and a blue glass filter.

Example. The recovery of 30 to 100 γ of

iodine added per egg containing 3 to 12 γ was 95 to 102%.

Colors; Preservatives

See Part I, C11, C12, and C13.

8. FOOD GELATIN

Chondrin

Rakusin Tests.[142] In the manufacture of gelatin from bones, the cartilage is removed as completely as possible, since otherwise the chondrin is extracted, causing an undesirable opalescence. The following tests may be applied.

Chrome Alum Test. To a hot 10% solution of the gelatin sample, add with continual stirring a *saturated solution of chrome alum* [$Cr(NH_4)(SO_4)_2 \cdot 12H_2O$]. In the presence of appreciable amounts of chondrin an immediate gelatinization takes place.

Opalescence Test. A solution containing 0.2% of chondrin in a test tube shows a milkiness sufficient to render the liquid decidedly opaque, whereas a 0.75% gelatin solution remains entirely clear. A gelatin solution may be titrated with the formation of glutinate.[143]

Protein Reactions. Of the eight known protein reactions, gelatin responds to only three (Biuret, Molisch, and Ostromyslenski), whereas chondrin in addition responds to the xanthoproteic reaction.

Barium Chloride Test. Of particular value is the reaction between *barium chloride* and chondroitinsulfuric acid present in both gelatin and chondrin. This substance is distinguished from sulfuric acid by its optical rotation ($[\alpha]_D = 46.5$). Because of the opalescence of chondrin solution, the barium chloride reaction is not visible, but if treated with *10% aluminum hydroxide solution*, to fix the protein rest, a clear solution is obtained which responds to none of the protein reactions, but reacts with barium chloride. The barium chloride test for chondroitinsulfuric acid may be applied directly to a solution of gelatin both as a qualitative test and for quantitative determination.

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I. ALKALOIDAL PRODUCTS

Tea, coffee, and cocoa are prized for their delectable flavors, as are also wines, malt liquors, and distilled liquors, yet the flavors are only psychological lures for the stimulation that man craves. Maté of South America and the cola nut of Africa both contain caffeine, but the betel nut of the Far East has other alkaloidal constituents.

Microscopic Structure (Figs. 181 to 186). Although the four alkaloidal beverage products pictured herewith all contain caffeine, and the cacao bean in addition theobromine, as their stimulating constituents, structurally the four products have little in common.

Coffee. The shelled coffee bean consists essentially of cells with knotty thickenings serving as the chief source of reserve material.

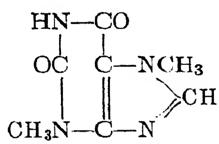
Cacao Bean. In addition to half its weight of fat, the shelled bean or nib contains about 10% of starch in rounded grains. Conspicuous in cocoa shells are groups of small stone cells from the stone cell layer, tangles of spiral vessels, large mucilage cells, and often yeast remaining from the fermentation of the beans.

Tea and Maté, in common with all leaves, have epidermal cells with stomata and chlorophyl-bearing tissues, but tea has in addition curious cane-shaped hairs and equally curious truss-like stone cells which hold the outer and inner epidermal layers well apart.

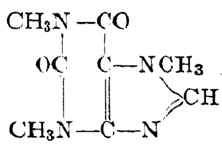
Chicory, although containing no alkaloid, is frequently added to coffee. It is a woody root with conspicuous vessels of the fibrovascular bundles and anastomosing latex tubes.

Adulteration. Although ingenious frauds, such as the manufacture of artificial coffee beans molded from dough with the addition of coloring or the less skillful addition of foreign leaves to tea in the country of production, are chiefly of historic interest, a thorough knowledge of the histology of alkaloidal products is still important. Only by microscopic examination can their identity in powdered form be established. Chocolate or cocoa is a common flavoring constituent in many powdered foods, as well as in confectionery, biscuits, and cake.

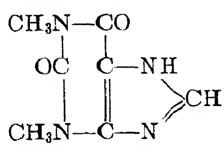
Alkaloidal Constituents. The purine bases *caffeine*, *theobromine*, and lesser known members of the group are related to the pyrimidine bases of which *cytosine* and *barbituric acid* are well-known examples. In the animal kingdom the best known members of the purine group are *purine*, *xanthine*, *adenine*, and *guanine* which are considered in the introduction to Part II, H.



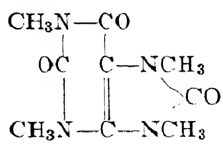
THEOBROMINE



CAFFEINE



THEOPHYLLINE



TETRAMETHYLURIC ACID

LEGENDS OF ILLUSTRATIONS ON FACING PAGE

The products are the leading alkaloidal foods and chicory. The magnification is ×160.

FIG. 181. Tea. Elements in surface view. Intervenal tissues: *aep* outer epiderm, *pal* palisade cells, mes^1 and mes^2 spongy parenchyma with vessels and rosette crystal cells, st^1 stone cell, and iep^1 lower epiderm with hair and stomata. Midrib tissues: st^2 stone cell, *fv* fibrovascular bundle elements, and iep^2 lower epiderm with hair.

The lance-like hairs of the epiderm and the large branching stone cells, often extending from the outer to the inner epiderm, cannot escape notice in the macerated tissue.

FIG. 182. Coffee. Elements in surface view. Fruit coat: *epi* epicarp with stomata, *f* crossing fibers, and *end* endocarp. (*pal* palisade cells in cross section.) *S* seed coat: sclerenchyma, parenchyma, and subepidermal cells with crystals. *P* perisperm with oil drops.

Sections of hard fragments of the bean show that the reserve matter is largely in the highly characteristic thick beaded cell walls. Large thick-walled, porous cells and thin-walled cells with minute crystals may be seen in the chaff from the cleft of the bean.

FIG. 183. Cocoa Nibs. Cotyledon in section: *ol* fat crystals, *pig* pigment cells, *ret* reticulated cells, *am* starch grains, and *al* aleurone grains. (*epc* epiderm in surface view.) *E* endosperm and *R* radicle in surface view.

The presence of starch grains distinguishes cocoa and chocolate from the other alkaloidal products illustrated. In some of the cells starch is replaced by dark-colored tannin. The curious broad hairs characterize the epiderm of the radicle.

FIG. 184. Cocoa Shells. Elements in surface view. Fruit coat: *mes* mesocarp pulp cells with *y* adhering yeast cells, *end* endocarp. Seed coat: *aep* outer epiderm, *p* spongy parenchyma, *sp* spiral vessels, and *sc* stone cell layer. (*muc* mucilage cells in cross section.)

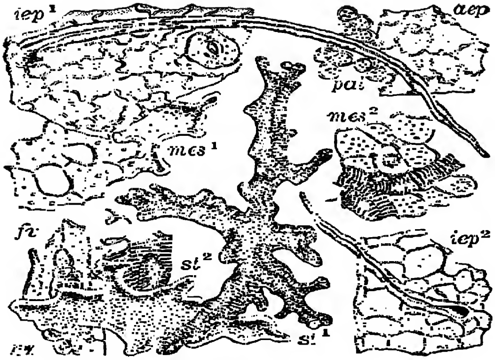
Tangled masses of spiral vessels characterize ground cocoa shells when present as an adulterant of cocoa products.

FIG. 185. Maté. Left, leaf in cross section; right, elements in surface view. *aep* outer epiderm, *pal* palisade cells, *ch* chain cells, *p* spongy parenchyma, *cr* crystals, *f* fiber, iep^1 inner epiderm over vein, and iep^2 inner epiderm between veins with stomata and water pore. *t* hair at base of leaf blade.

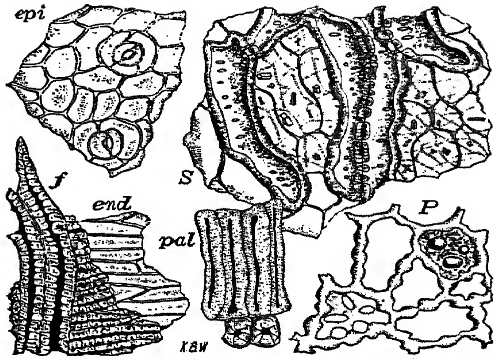
The water pores and the beaded cells with short warty hairs distinguish maté from tea. Oxalate aggregates occur in both leaves.

FIG. 186. Chicory Root. *su* cork in tangential and cross section. Longitudinal radial section in alcoholic material: *v* reticulated vessels, *w* wood fibers, *xp* xylem parenchyma, *l* latex system, *s* sieve tubes, *m* medullary ray, and *p* phloem with *in* inulin crystals.

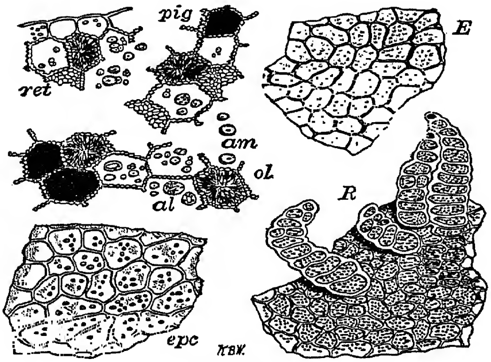
Broad pitted or reticulated vessels (characteristic of root tissues), latex cells, and inulin that crystallizes in alcoholic preparations are the most noticeable microscopic elements.



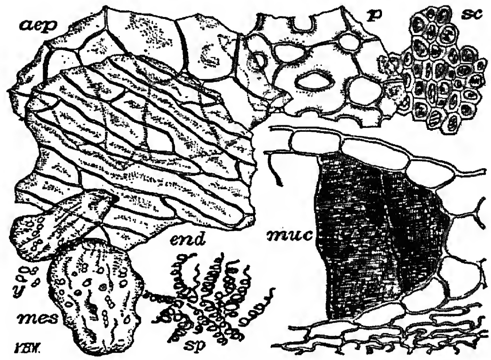
Tea



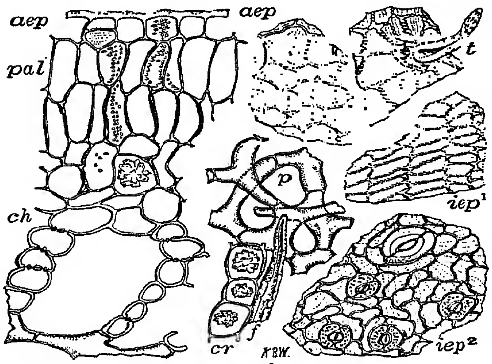
Coffee



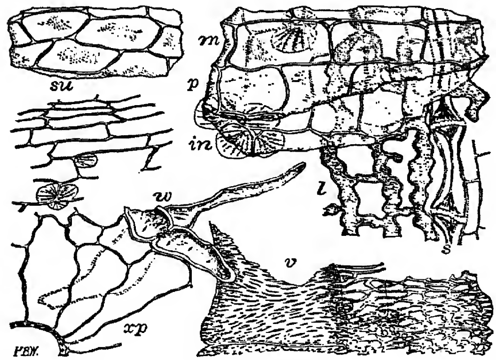
Cocoa Nibs



Cocoa Shells



Maté



Chicory

FIGS. 181–186.

1. TEA

The different varieties of tea are classed under one species, *Thea sinensis* L., a native of the Far East. Although tea has been grown successfully in South Carolina, the world's supply is still produced in China, India, Japan, and neighboring regions. *Green tea* owes its color to chlorophyl that is preserved by steaming, thus rendering enzymes inert, and immediately drying. *Black tea* is subjected to a fermentation process that destroys the chlorophyl and produces other changes.

Microscopic Structure. See above.

Chemical Composition. Analyses by Geisler,[1] Spencer,[2] and Tatlock and Thomson [3] show the following range:

Pierotti [5] found, in addition to the usual constituents, 4.04 to 6.97% of dextrin and gums in commercial tea.

Analytical Methods. The analysis of tea serves partly to show the content of alkaloids, tannin, and other valuable constituents and partly to detect adulteration.

Water; Protein; Fat; Fiber; Ash; Water-Soluble Ash; Acid-Soluble Ash; Alkalinity of Ash

Water (moisture) and protein are determined by methods given under Coffee, below; fat, fiber, ash, water-soluble ash, acid-insoluble ash (sand), and alkalinity of ash as directed in Part I, C2, 5, and 7.

	Water	Caffeine	Tannin	Total Water Extract	Half-Hour Water Extract	Ash		
						Total	Water-soluble	Sand
	%	%	%	%	%	%	%	%
Min.	3.59	1.00	4.77	27.48	23.48	5.14	1.66	0.03
Max.	9.90	4.91	20.07	54.36	45.28	8.91	5.02	2.74

The wide range in percentages of constituents in analyses by Kellner, Makino, and Ogasawara,[4] given in the following table, is due chiefly to the time of picking; protein, caffeine, and nitrogen-free extract decrease, whereas fat, tannin, fiber, and ash increase as the season advances.

Caffeine

Power and Chesnut Ethanol-Chloroform Extraction Gravimetric Method.[6] Process. *Ethanol Extraction.* Extract 10 g. of the finely ground sample, previously moistened

	Water *	Protein †	Caffeine	Fat ‡	Nifext §	Tannin	Water Extract	Fiber	Ash
Min.	59.43	14.19	1.00	6.48	37.29	8.53	30.01	9.10	4.29
Max.	76.83	21.50	2.85	22.19	49.49	12.16	38.21	19.16	5.04

* When picked. † (Total N less caffeine N) $\times$ 6.25. ‡ Ether extract. § Includes tannin.

with *ethanol*, for 8 hours in a Soxhlet extractor with hot *ethanol*.

Magnesium Oxide Treatment. Suspend 10 g. of *heavy magnesium oxide* in 100 ml. of water in a porcelain dish and add to the suspension the ethanolic extract, rinsing with a little hot water. Evaporate the mixture slowly on the steam bath with frequent stirring until all the ethanol is removed and a nearly dry powdery mass is obtained. Mix with the mass hot water sufficient to permit transferring to a smooth filter and wash with hot water until the filtrate collected in a 1-liter flask measures about 250 ml.

Acid Hydrolysis. To the filtrate, add 20 ml. of *1 + 9 sulfuric acid* and heat, at first gently until frothing ceases, then boil for 30 minutes. By this treatment, saponins, which may be present in certain leaf products for which the method is adapted, are hydrolyzed. Cool, filter through a moistened double paper into a separatory funnel, and wash with small portions of *0.5% sulfuric acid*.

Chloroform Extraction. Shake out the clear acid filtrate with six successive 25-ml. portions of *chloroform*, gently rotating or adding a somewhat larger portion of the solvent if the separation is not sharp.

Alkali Treatment. To the united chloroform extracts contained in a separatory funnel, add 5 ml. of *1% potassium hydroxide solution* and shake to remove color. Draw off the chloroform solution onto a small dry paper and collect the filtrate in an Erlenmeyer flask. Wash the alkaline liquid with two portions of *chloroform* of 10 ml. each, passing each through the paper, and finally wash the paper with a little *chloroform*.

Evaporation. Remove the chloroform from the united chloroform solution and washings by distillation and transfer to a tared beaker with the aid of *chloroform*. Allow the solvent to evaporate spontaneously, dry for 30 minutes in a boiling water oven, and weigh the anhydrous caffeine. Repeat the heating and weigh again.

Calculation. Multiply the final weight by 10 to obtain the percentage of caffeine.

Bailey and Andrew Chloroform Extraction Gravimetric Method.[7] In this procedure, the sample is treated directly with magnesium oxide, omitting the initial extraction with ethanol employed in the Power and Chesnut method.

Process. *Magnesium Oxide Treatment.* Place 5 g. of the sample ground to pass a 30-mesh sieve in a 500-ml. flask, add 10 g. of *heavy magnesium oxide* and 200 ml. of water, and boil gently for 2 hours over a low flame, using a small-bore glass tube 30 inches long as condenser. Cool, make up to volume, and filter through a dry paper.

Acid Hydrolysis. Pipet an aliquot of 300 ml. into a 1-liter Erlenmeyer flask, add 10 ml. of *1 + 9 sulfuric acid*, and boil down to about 100 ml. Filter into a separatory funnel, rinsing with small portions of *1 + 99 sulfuric acid*.

Chloroform Extraction. Shake with successive 25-, 20-, 15-, 10-, 10-, and 10-ml. portions of *chloroform*.

Alkali Treatment. Shake the combined extracts with 5 ml. of *1% potassium hydroxide solution* and draw off the chloroform layer into a flask. Wash the alkaline solution twice with 10 ml. of *chloroform* and unite with the main extract.

Evaporation. Evaporate the combined chloroform extracts to small bulk, transfer to a weighed flask, rinsing with *chloroform*, evaporate to dryness, and dry in an oven to constant weight.

Determine the nitrogen and multiply by the factor 3.464 to obtain the percentage of anhydrous caffeine.

Tannin

Löwenthal-Procter Permanganate Volumetric Method.[8] Reagents. *Standard Potassium Permanganate Solution.* Dissolve

1.33 g. of $KMnO_4$ in water and dilute to 1 liter. Standardize by titrating against 0.1 N oxalic acid solution.

Oxalic Acid Solution. 0.1 N. Dissolve 6.3 g. of the acid ($C_2H_2O_4 \cdot 2H_2O$) in water and dilute to 1 liter.

Indigo Carmine Solution. Dissolve 6 g. of indigo carmine (free from indigo blue) and 50 ml. of H_2SO_4 in water and dilute to 1 liter.

Gelatin Solution. Soak 25 g. of gelatin for 1 hour in saturated NaCl solution, heat until the gelatin dissolves, cool, and dilute to 1 liter.

Acid-Salt Mixture. Mix 975 ml. of saturated NaCl solution and 25 ml. of H_2SO_4.

PROCESS. *Extraction.* To 5 g. of the powdered sample in a 500-ml. volumetric flask, add 400 ml. of cold water and boil for 30 minutes. Cool, make up to the mark, and filter on a dry paper.

Direct Permanganate Titration. To 10 ml. of the filtrate, add 25 ml. of *indigo carmine solution* and 750 ml. of water, then *potassium permanganate solution,* with vigorous stirring, 1 ml. at a time until the liquid becomes light green in color. Continue adding *permanganate solution* dropwise until the color changes to a golden yellow (Councler and Schroeder) [9] or a pure yellow color with a pinkish rim (Procter). Record the buret reading (A), which is the amount of permanganate solution required to oxidize all oxidizable substances.

Permanganate Titration after Gelatin Treatment. Pipet 100 ml. of the tea extract into an Erlenmeyer flask, add 50 ml. of *gelatin solution* and 100 ml. of the *acid-salt mixture,* both measured from pipets, then 10 g. of *kaolin.* Cork and shake vigorously for several minutes. Allow the precipitate to settle and decant the supernatant liquid on a dry paper. Mix 25 ml. of the filtrate (equivalent to 10 ml. of the extract) with 25 ml. of *indigo carmine solution* and 750 ml. of water, then titrate with *permanganate solution* as before. Record the reading (B), which is the amount of permanganate solution required to oxidize the substances other than tannin.

CALCULATION. Subtract B from A, thus obtaining the number of milliliters of permanganate solution required to oxidize the tannin. Calculate the equivalent number of milliliters of 0.1 N oxalic acid solution and multiply by 0.004157. The product is the corresponding weight of gallotannic acid or tannin.

NOTE. Barua and Roberts [10] regard the Stamm alkaline potassium permanganate method as more accurate than other current methods since the tannins are completely oxidized to carbon dioxide, whereas the other methods are suitable only for unoxidized tannins.

WATER EXTRACT

The percentage of extract (hot water extract) is the complement of the percentage of the insoluble leaf; given either and the percentage of water, the other may be calculated.

As originally carried out by Spencer [11] and by later authors,[12] the residue, after hot water extraction, was dried and weighed, then from the weight the percentage of residue (insoluble leaf) was calculated. The purpose was to detect the presence of exhausted tea, then a rare and now an obsolete adulterant.

At present the preferred practice is to evaporate an aliquot of the filtrate from the insoluble leaf and from its weight calculate the percentage of extract, since this is more useful than the percentage of insoluble leaf in supplementing organoleptic tests for determining grade or quality or in securing uniformity of an advertised brand. Other aliquots of the filtrate may be used for determinations of its various constituents, especially caffeine.

Official Evaporation Method.[13] Add to 2 g. of the ground sample, contained in a 500-ml. volumetric flask, 200 ml. of hot water and boil gently for 1 hour with occasional rota-

tion. Appreciable loss of moisture is prevented by attaching a perforated rubber stopper through which passes a 30-inch (75-cm.) glass tube that acts as a condenser. Cool, dilute to the mark, mix, and filter through a dry paper. Evaporate an aliquot of 50 ml. to dryness, dry at 100° for 1 hour, cool, and weigh.

Facing

Chinese and Japanese tea, prepared for export, were formerly faced or coated to increase the weight or improve the appearance or both. Among the facing materials for black tea, plumbago is most often mentioned; the list for green tea is more extensive, including graphite, gypsum, Prussian blue, ultramarine, indigo, and turmeric.

Facing is detected by microscopic examination of the siftings and the application of micro tests to individual particles separated under a lens. Plumbago has a dark metallic luster, gypsum is white, and soapstone greenish gray. The blue pigments are not distinguishable in microscopic particles by the shade of color, but by color reactions. Leach and Winton [14] note that Prussian blue is decolorized by sodium hydroxide solution, whereas ultramarine and indigo are not. Ultramarine is decolorized by hydrochloric acid but indigo is not. Indigo, although not affected by sodium hydroxide solution or hydrochloric acid, becomes yellowish with sulfuric acid, changing slowly to blue-green. Only when a particle is freed from the accompanying fragments of organic matter that would be charred by the concentrated acid is the sulfuric acid test applicable.

Read [15] describes an ingenious test suited for the use of inspectors without chemical training. The siftings are mashed down on white paper with a stroke of a spatula; thus pigments of different shades of blue produce streaks of corresponding tints. If gypsum or other light-colored facing materials are present, dark paper is substituted for white.

2. COFFEE

Although the scientific name is *Coffea arabica* L., the coffee tree, of which the coffee bean is the berry, originated in Abyssinia and adjoining regions of Africa. At present Brazil leads the world in coffee production.

Coffee beans are decorticated in the countries of production and roasted on a large scale by the distributors. The grinding to different degrees of fineness is performed by the distributor, the retailer, or the consumer.

Microscopic Structure. See first page of Part II, I.

Chemical Composition. Analyses by Lythgoe [16] of roasted Santos, Porto Rico, Rio, Mocha, and Java coffee, each of three grades, show the following composition:

	Water	Protein *	Caffeine	Fat	Reducing Sugars	Starch †	Cold Water Extract	Hot Water Extract ‡	Ash		
									Total	Water-soluble	Alkalinity §
	%	%	%	%	%	%	%	%	%	%	
Min.	1.26	10.50	1.10	12.28	0.32	1.00	20.27	24.60	3.74	3.00	71.4
Max.	3.44	13.69	1.38	15.18	1.78	3.34	24.44	27.70	4.38	3.62	102.3
Aver.	2.16	12.19	1.20	13.75	0.75	2.30	22.63	27.20	4.03	3.26	87.1

* (Total N less caffeine N) × 6.25. † Results by the diastase method; no starch is evident under the microscope. ‡ Calculated from solids of 10% extract. § Milliliters of 0.1 N acid per ash of 1 g. of sample.

Fat; Sugar; Fiber; Ash

Most of the proximate constituents, : bly fat, sugar, fiber, and ash (total, soluble, insoluble, and alkalinity), may be determined by the methods given under General Methods, Part I, although the occasions for such determinations are not so frequent as in the case of cereal products, since only those constituents that go into solution in the preparation of the beverage are of value to the consumer. The methods for the constituents which follow are of special significance.

Water

Moisture plays an important role in commercial or regulatory controversies over the weight of wholesale shipments or retail pack-

Since coffee beans, as well as cocoa beans and tea, are roasted or fired before they reach the consumer and tend to increase, rather than decrease, in moisture content during handling, a uniform method for the three products, employing a heat higher than that of the boiling water oven, seems desirable. Such a uniform method could be used also for the raw products.

The Tentative A.O.A.C. Method specifies for coffee drying for 5 hours at the temperature of boiling water under atmospheric pressure or at 105 to 110° under reduced pressure (100 mm. of mercury), for cocoa drying in an air oven at 100° to constant weight, and for tea drying at 95 to 100° under reduced pressure for 5 hours.

Differences in details of conventional methods for different classes of products adopted by different groups working independently are not necessarily indicative of the needs of special treatment for each group. The writers venture to suggest a further extension of the Association's wisely directed efforts at uniformity. A uniform method for the three alkaloidal products, raw or roasted, also perhaps for maté and cola nuts, such as drying at 105 or 110° (not 105 to 110°) in a well-regulated air oven, would appear to meet the requirements. Drying *in vacuo* or in hydrogen seem superfluous since the products already have been exposed to oxidation in preparation.

Caffeine

Opinions differ as to whether the caffeine in coffee exists free or combined with chlorogenic acid. In some of the methods the material is digested with magnesia or ammonium hydroxide preliminary to extraction; in others it is extracted directly with one of the several solvents. Notwithstanding these differences, most if not all the methods appear to separate the alkaloid from the raw or roasted coffee in about the same amount and in a reasonable degree of purity; the Gorter method, however, has been shown by Murray [17] to give results on decaffeinated coffee containing less than 0.5% of caffeine, 50 to 100% higher than the Lendrich and Nottbohm method.

Gorter Chloroform Extraction Method.[18] The method was devised at the laboratory of the Department of Agriculture, Buitenzorg, Java.

Process. Extract for 3 hours in a Johnson or Soxhlet extractor with *chloroform* 11 g. of the finely powdered sample which previously has been moistened with 3 ml. of water and allowed to stand 30 minutes. Evaporate the extract to dryness, take up the residue of fat and caffeine with hot water, filter through a cotton pledget and a moistened paper into a 55-ml. volumetric flask, wash with hot water, and make up to the mark. Pipet 50 ml. of the liquid into a separatory funnel and shake out with four portions of *chloroform*. Evaporate the combined chloroform extracts in a tared dish, dry at 100°, and weigh the caffeine.

Remove the weighed caffeine to a Kjeldahl

flask by means of *chloroform*, rinse with *Kjeldahl sulfuric acid*, and determine the percentage of nitrogen by the Kjeldahl-Wilfarth or Kjeldahl-Gunning method. Multiply by the factor 3.464 to obtain the corresponding percentage of anhydrous caffeine.

Lendrich and Nottbohm Carbon Tetrachloride Extraction Method.[19] REAGENT. *Hydrogen Peroxide-Acetic Acid Mixture.* One milliliter of 96% acetic acid in each 100 ml. of 3% hydrogen peroxide solution.

PROCESS. *First Extraction.* Grind the sample to pass a 1-mm. mesh sieve. Add to 20 g. of the ground coffee 10 ml. of water and stir at intervals during 1 to 2 hours, then extract in a Soxhlet extractor for 5 to 8 hours with *carbon tetrachloride.* To the extract, add 1 g. of *solid paraffin*, distil the carbon tetrachloride, and treat the residue with one portion of 50 ml. and three portions of 25 ml. of boiling water. After cooling, filter the combined extracts through a moistened paper, and wash with hot water.

Oxidation. Cool the aqueous liquid (about 200 ml.) to room temperature, add 10 ml. (30 ml. for roasted coffee) of *1% potassium permanganate solution*, and after 15 minutes precipitate the excess of manganese with a little *hydrogen peroxide-acetic acid mixture* (usually 2 to 3 ml. for roasted, more for raw) added dropwise. Heat 15 minutes on a steam bath, filter, wash with boiling water, evaporate the filtrate to dryness, and dry in a boiling water oven for 15 minutes.

Second Extraction. Take up the warm residue with warm *chloroform* and filter into a tared dish, washing with *chloroform.* Evaporate the chloroform, dry the residue for 30 minutes in a boiling water oven, cool, and weigh the anhydrous caffeine.

Transfer the caffeine to a Kjeldahl flask, determine the nitrogen, and calculate the anhydrous caffeine by the factor 3.464.

EXAMPLES. Results by Lendrich and Nottbohm [20] and by Nottbohm and Mayer [21] on coffee grown in various countries follow: raw, 0.96 to 2.60; roasted, 1.15 to 3.00% of caffeine.

I. Fendler and Stüber Ammonia-Chloroform Extraction Modification.[22] REAGENT. *Hydrogen Peroxide-Acetic Acid Mixture.* As for Lendrich and Nottbohm Method.

PROCESS. *Ammonia-Chloroform Extraction.* Place 10 g. of the sample, ground to pass a 1-mm. mesh sieve, 10 ml. of *10% ammonium hydroxide*, and 200 g. of *chloroform* in a glass-stoppered bottle, shake vigorously and continuously for 30 minutes, then filter through a pleated paper large enough to hold the whole mixture. In these operations, take care to avoid evaporation by exposure and, as directed in the *A.O.A.C. Methods* (1940), keep both flask and receiver in an ice bath. Evaporate 150 g. of the filtrate in a wide-mouth 250-ml. flask on a boiling water bath, employing an air blast to remove the last traces of chloroform.

Permanganate Oxidation. Add to the residue 80 ml. of hot water, heat on a boiling water bath with frequent shaking for 10 minutes, and cool, then add 20 ml. (for roasted coffee) or 10 ml. (for raw coffee) of *10% potassium permanganate solution.*

Peroxide Treatment. After standing at room temperature for 15 minutes, remove the excess of permanganate by adding 2 ml. of *hydrogen peroxide-acetic acid mixture*, followed if still red by 1 ml. additional and still more if the red color persists. Heat on a boiling water bath with addition of 0.5-ml. portions of the *mixture* until there is no further change in color, continuing the heating to a total of 15 minutes. When cool, filter on a moistened 9-cm. paper, and wash with cold water up to about 200 ml.

Chloroform Extraction. Shake the clear filtrate with 50 ml. of *chloroform*, followed by three portions of 25 ml. Evaporate the combined extracts in a wide-mouth 250-ml. flask, removing the last traces of chloroform by an air blast as before. Dry the caffeine thus obtained at 100° to constant weight.

II. Grossfeld and Steinhoff Ammonia-Carbon Tetrachloride Extraction Modification.[23] As used at the Berlin Food Inspection Laboratory, the technique is as follows.

PROCESS. *Extraction.* To 10 g. of the finely ground sample, add 5 ml. of *10% ammonium hydroxide*, mix, and allow to stand 1 hour (2 hours for raw coffee) with occasional stirring. Use 20 g. of decaffeinated coffee and double the amount of the reagents. Add 10 to 15 g. of *coarse sand*, filter on a pleated paper, and extract with *carbon tetrachloride* for 2 hours in a continuous extraction assembly. Remove the solvent from the extract, transfer to a 200-ml. volumetric flask, rinse with hot water until about three-fourths full, and cool. (In the case of decaffeinated coffee, add 1 drop of *paraffin*, evaporate, and treat the residue with 50 ml. of water, then bring to a boil and cool.)

Permanganate Treatment. Add 10 ml. of *5% potassium permanganate solution* and allow to stand 15 minutes.

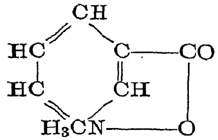
Thiosulfate Treatment. Add 5 ml. of *20% copper sulfate solution* and remove the excess of permanganate with 5 ml. of *10% sodium thiosulfate solution*, then add 5 ml. of *normal ammonium hydroxide*, make up to the mark, and filter through a dry pleated paper.

Chloroform Extraction. Shake 100 ml. of the filtrate with 50 ml., then with three 25-ml. portions of *chloroform*, filter the combined extracts through a pleated paper into a tared flask, wash with *chloroform*, distil off the solvent, and dry the flask on its side to constant weight in a steam bath.

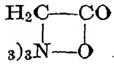
Power and Chesnut Ethanol-Chloroform Extraction Method.[24] See Tea above.

TRIGONELLINE

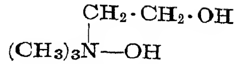
According to Nottbohm and Mayer (Hamburg), coffee contains in addition to caffeine, three bases, trigonelline,[25] betaine,[26] and choline:[27]



TRIGONELLINE



BETAINE



CHOLINE

Analyses by these authors show up to over 0.24% of trigonelline and about 0.02% of choline, the former being determined in the residue after the extraction with chloroform in the determination of caffeine. They found that a low caffeine content is associated with a high trigonelline content and vice versa. In preparing an aqueous infusion, 81.49% of the caffeine and 69.44% of the trigonelline were removed. They further stated that trigonelline is not removed in the decaffeinating process.

Nottbohm and Mayer Iodometric Method.[28] PROCESS. *Chloroform Extraction.* Extract 20 g. of the sample with *chloroform*, in which trigonelline is insoluble, as in the Lendrich and Nottbohm method for the determination of caffeine.

Ethanol Extraction. Extract the residue for 3 hours with *ethanol*, adding a new portion of the *ethanol* after the first hour.

Lead Precipitation. Add 8 ml. of *lead subacetate solution* to the combined ethanolic extracts, collect the orange-yellow precipitate of lead chlorogenate on a large paper, and wash with *ethanol*.

Deleading. Precipitate the lead in the filtrate with *hydrogen sulfide*, filter, and wash.

Sugar Destruction. Evaporate the filtrate and washings to 50 ml. with the addition of 5 ml. of *25% hydrochloric acid*, filter to remove the oil that separates, evaporate the filtrate with a little *charcoal*, take up with water, add a few drops of the *acid*, and evaporate to dryness. Repeat the addition of wa-

ter and *charcoal*, then filter, wash with water, and evaporate to 5 ml.

Iodine Precipitation. Add 3 drops of *25% hydrochloric acid* and 11 ml. of *0.1 N iodine solution.* The iodine compound from raw coffee forms steel-blue needles; that from roasted coffee is an oily substance of the same composition (1 molecule of trigonelline to 3 molecules of iodine). Collect the precipitate on a Gooch crucible or sugar tube after 10 minutes, or when the brown clouds have settled completely, wash with cold water, then dissolve in warm *ethanol.*

Thiosulfate Titration. Dilute the solution and titrate with *standard 0.1 N sodium thiosulfate solution.*

CALCULATION. Use the equation: 1 ml. of 0.1 *N* thiosulfate solution = 4.9 mg. of trigonelline.

Nottbohm and Mayer Modification.[28] PROCESS. *Water Extraction.* After the preliminary extraction with chloroform, extract the residue with 150 ml. of water on a boiling water bath, then extract by boiling with 150 ml. of water over a low flame. Decant the liquid and evaporate to about 150 ml.

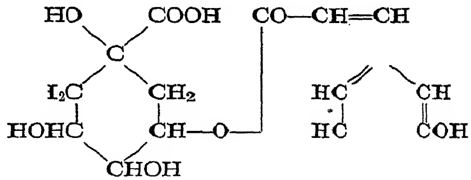
Removal of Sugar. To the liquid (which need not be clear), add 25 ml. of *25% hydrochloric acid* and autoclave at 4.5 atmospheres. Filter from the carbonaceous matter, clarify the filtrate with *animal charcoal,* concentrate in vacuum, and extract the residue three times with 20 ml. of boiling *ethanol,* filtering each time through a hot water funnel. Evaporate the ethanol extract to dryness, take up in 25 ml. of water, clarify with a pinch of *animal charcoal,* filter, evaporate to about 5 ml. and proceed with the iodometric precipitation and titration as in the original method.

CHOLINE

Nottbohm and Mayer Iodometric Method.[29] The authors describe a lengthy process for the determination of choline in the ethanolic extract prepared as for the determination of trigonelline. The operation includes autoclaving with *hydrochloric acid* to destroy sugars, decolorizing with *charcoal,* clarification with *phosphotungstic acid,* removal of the excess of the latter by *baryta water,* and iodometric titration by the Roman method.[30] The amount of choline reported was only 0.02%. A study of recoveries of added amounts of choline seems desirable before placing dependence on the method.

CHLOROGENIC ACID

Gorter[31] demonstrated that caffetannic acid is a mixture consisting of chlorogenic acid and coffalic acid, a new substance. As advanced by Freudenberg[32] and confirmed by Fischer and Dangschat,[33] the formula for chlorogenic acid, showing its quinic acid (left) and caffeic acid (right) components, is as follows:



Jurany Lead Precipitation Polarimetric-Volumetric Method.[34] The method was devised at the Provencial Laboratory at Bremen.

APPARATUS. *Polariscope.*

PROCESS. *Ethanol Extraction.* Mix 7 to 8 g. of very finely ground raw coffee, or 15 to 20 g. of ground roasted coffee, with an equal weight of *fine quartz sand.* Introduce the mixture into the inner tube of a Johnson extractor or the paper thimble of a Soxhlet extractor and extract with 70 ml. of *80% ethanol* for 4 hours. Dry the residue at 50 to 70° and grind with the sand in an agate mortar. (Omit the grinding if the coffee is roasted.) Add an equal weight of *coarse quartz sand* and

repeat the extraction until a portion of the ethanol extract gives no precipitate with *lead acetate solution.* Add to the combined extracts 2 g. of *solid paraffin* to bind the fat and wax, and evaporate to 50 ml. Cool, break up the paraffin, filter through a pleated paper into a 150-ml. volumetric flask, and wash with water. The filtration proceeds slowly and the filtrate is not always clear, but a slight turbidity is not detrimental.

Lead Chlorogenate Precipitation. Transfer a large aliquot to a 400-ml. beaker, add 20 ml. of *ethanol* and water to a total volume of 200 ml., and heat to 80 to 90°, then slowly add 12 to 15 ml. of *25% lead acetate solution.* Heat 10 minutes over a small flame and cool in ice water for 1 hour. Collect the lemon yellow lead salt of chlorogenic acid on a smooth filter and wash until the filtrate gives only a slight reaction for lead.

Chlorogenic Acid Liberation. Rinse the precipitate from the paper into the beaker previously used with not more than 100 ml. of hot water and saturate the mixture with *hydrogen sulfide gas,* thus precipitating the lead and liberating the chlorogenic acid. Continue the flow of *hydrogen sulfide* until the precipitate is jet black and no more yellow particles are visible, then filter, transferring both the liquid and precipitate to the paper. To the beaker thus emptied, transfer the paper on which the lead chlorogenate had been filtered, beat to a pulp with a small amount of water, and use the liquid that separates to wash the lead sulfide precipitate, repeating the operation if necessary.

Remove the excess of hydrogen sulfide from the filtrate by means of a stream of air, then evaporate to a bulk of less than 100 ml., transfer to a 100-ml. volumetric flask, make up to the mark (or make up to 200 ml. without evaporation), mix, and use the solution for polarization and titration.

Polarization. Obtain the angular rotation of the solution, using a 20-dm. tube and calculate the weight of optically active substance (W) in 100 ml. of the solution by the following formula:

$$W = \frac{100 \times \alpha}{[\alpha]_D \times l} = \frac{100 \times \alpha}{662}$$

in which α is the reading in circular degrees, $[\alpha]_D$ is the specific rotation of the optically active substance (= 33.1°), and l is the length of the tube in decimeters (= 20).

Titration. Pipet a suitable aliquot of the solution into a beaker and titrate with *0.1 N sodium hydroxide solution,* using a few drops of *bromothymol blue solution* as indicator and taking the reading when the color changes from yellow to emerald green with a bluish tint. The molecular weight of chlorogenic acid is 363.23.

Chlorogenic Caffeic Acids

Hoepfner Alkaline Nitrite Colorimetric Method.[35] Hoepfner (Hamburg) demonstrated that (1) *caffeic acid* in acetic or phosphoric acid solution gives with an alkali nitrate an intense cinnabar red coloration (orange in highly dilute solution), the sensitivity being 1:100,000, and (2) *chlorogenic acid,* by the same treatment, gives an intense yellow solution, changing with an excess of sodium hydroxide (owing to the conversion into caffeic acid) to an intense carmine red. He declares that, in both tests, addition of urea prevents the formation of nitrogen dioxide which destroys the color.

Apparatus. *Pulfrich Photometer.*

Process. A. Chlorogenic Acid Plus Caffeic Acid. *Water Extraction.* Defat 5 g. of the finely ground raw coffee beans by boiling for about 30 minutes with *acetone.* Filter, dry the fat-free residue, and soak for some hours in 50 ml. of *concentrated sodium chloride solution,* then dilute to 100 ml. and boil for 15 minutes. Decant, add 50 ml. of water and boil again for 15 minutes, repeating the treatment four or five times for complete extraction. Adjust the combined ex-

tract to exactly 250 ml., allow to settle, and filter while still warm.

Color Formation. Dilute an aliquot of 5 ml. of the extract with 25 ml. of water and add 0.5 ml. of *saturated sodium nitrite solution* and 7 g. of *urea*. Then add with shaking 0.5 ml. of *glacial acetic acid,* followed after 3 minutes by 5 ml. of *sodium hydroxide solution,* added quickly, and dilute to 100 ml.

Color Measurement. Measure the absorption (due to caffeic acid) in a 1-cm. cell of the Pulfrich photometer, using light filter 610 or 570 mμ wave length.

Compare the absorption with that of an aliquot of 5 ml. of a solution of 0.1 g. of chlorogenic acid (= 0.164 g. of potassium caffeine chlorogenate) treated in like manner with sodium nitrite, urea, acetic acid, and sodium hydroxide.

In raw coffee the result for most purposes may be regarded as representing chlorogenic acid, since caffeic acid is assumed not to be present in appreciable amount.

B. Caffeic Acid. In the analysis of coffee variously treated, determine the caffeic acid as under A, omitting the addition of alkali.

C. Chlorogenic Acid. Subtract B from A.

I. Plücker and Keilholz Direct Acetate Buffer Modification.[36] The color is formed directly in the water extract with the addition of an acetate buffer solution. Urea is omitted. The modification gives accurate results in raw coffee agreeing with those of the Hoepfner method, but when applied to roasted coffee the average is about 20% high.

Reagent. *Acetate Buffer Solution.* Dissolve 10 ml. of glacial acetic acid and 30 g. of sodium acetate in water and dilute to 100 ml.

Process. *Water Extraction.* Weigh 5 g. of the finely ground sample into a 100-ml. volumetric flask, add 80 ml. of boiling water, boil gently for 30 minutes, avoiding loss by foaming, cool, make up to volume, mix, and filter through a dry paper, avoiding evaporation. (Use an aliquot of this extract also for Modification II below.)

Color Formation. Pipet 10 ml. of the water extract into a 100-ml. volumetric flask, make up to the mark, mix, and pipet 2 ml. of the solution into a test tube. Dilute with 8 ml. of water, add 0.3 ml. of *acetate buffer solution,* and cool to 8°. Add 0.3 ml. of *40% sodium nitrite solution* at 8°, then after exactly 3 minutes at 8° add quickly 0.3 ml. of *10% sodium hydroxide solution,* all measurements being made from burets or pipets.

Color Measurement. Place the mixture of the 2 ml. of the diluted extract plus reagents in the left-hand 1-cm. cell of the Pulfrich photometer and a mixture of 2 ml. of the diluted extract plus 8.9 ml. of water (8 + 0.3 + 0.3 + 0.3) in the right-hand 1-cm. cell. Determine the percentage of color transmission (D), using filter S 53 or S 57.

Calculation. Obtain the extinction coefficient (K) by the formula $K = \log (100/D)$, then obtain K_1 from the table below and calculate the percentage of chlorogenic acid (P) as follows:

$$100 \times \frac{100}{10} \times \frac{10}{2} \quad \frac{1}{1000} \times \frac{100}{5}$$

$$0.964K$$

in which 96.4 is the volume of solution in the 100-ml. volumetric flask corrected for the volume of insoluble matter.

D	K_1 (Filter S 53)	K_1 (Filter S 57)
0	0.192	0.115
10	0.186	0.110
20	0.180	0.105
30	0.174	0.100
40	0.167	0.095
50	0.159	0.090
60	0.148	0.085
70	0.137	0.078
80	0.125	0.070
90	.....	0.061

If desired, calculate the result to the fat- and water-free basis. Plücker and Keilholz assume that 6.51% of fat and 7.71% of water (total 14.22%) are present in raw coffee.

II. Plücker and Keilholz Indirect Acetate Buffer Modification.[37] The water extract is hydrolyzed and caffeic acid is extracted with ether preliminary to color formation as in Modification I. Accurate results are obtained on chlorogenic acid in roasted as well as raw coffee.

PROCESS. *Water Extraction.* Prepare a water extract as in Modification I.

Alkali Hydrolysis. To 50 ml. of the water extract at 18°, add exactly 12 ml. of *30% potassium hydroxide solution* and allow to stand 1 hour at 18°, then add slowly with cooling 10 ml. of *1 + 2 sulfuric acid* and filter.

Ether Extraction. Transfer 50 ml. of the filtrate to a separatory funnel, shake once with 100 ml. of *ether*, draw off and discard the aqueous layer, and wash the ether layer with three portions of 25 ml. each of water. Transfer the ether solution to a small flask, add 50 ml. of water, and distil until the ether layer has been driven over. Cool the residual aqueous solution in the flask, transfer to a 100-ml. volumetric flask, and dilute to the mark.

Color Formation. Remove 1 ml. of the extract with a pipet, dilute with exactly 9 ml. of water, add 0.15 ml. of *acetate buffer solution* (see Modification I) and 0.15 ml. of *40% sodium nitrite solution*, all measured at 18° (total volume $1 + 9 + 0.15 + 0.15 = 10.3$ ml.), then allow to stand 10 minutes at the same temperature.

Color Measurement. Place in the left-hand 1-cm. cell of the Pulfrich photometer the mixture of the solution of the unknown plus reagents, then place in the right-hand 1-cm. cell 1 ml. of the solution of the unknown plus 9.3 ml. of water. Determine the percentage of color transmission (D), using filter S 53.

CALCULATION. Calculate the extinction coefficient (K) by the formula $K = \log(100/D)$. Obtain K_1 and the conversion factor (F) from the table below and calculate the percentage of chlorogenic acid (P) in the sample by the following formula:

$$P = \frac{K}{K_1} \times 50 \times \frac{10}{1} \times \frac{1}{1000} \times \frac{100}{5}$$

$$\frac{0.3856K \times F}{K_1}$$

D	K_1	K/K_1	F
0	0.287	0	4.95
10	0.290	10	4.79
20	0.293	20	4.53
30	0.296	30	4.47
40	0.299	40	4.31
50	0.302	50	4.15
60	0.305	60	4.00
70	0.308	..	....

The average of results obtained by Plücker and Keilholz in a sample of raw coffee, using filters S 53, S 47, and S 57, is 7.176% of chlorogenic acid which differs only 0.048% from the result when filter S 53 is used. All results are reduced by them to the fat- and water-free basis as noted under Modification I above.

EXAMPLES. Comparative results by the three methods for the determination of chlorogenic acid, as obtained by Plücker and Keilholz below.

WATER EXTRACT

Coffee and tea are unique in that only the hot water extract is valuable to the consumer and the grounds or spent leaf are waste products. Examination of the water extract falls chiefly to the taster, not the chemist. Correlation between flavor, strength, and other organoleptic properties and chemical composition of the extract are not marked well, if at all. The nutritionist, however, takes

	1 Hoepfner Method	2 Acetate Buffer Mod. of the Hoepfner Method	3 P. and K. Caffeic Acid Method	Difference, Methods 2 and 3
	%	%	%	%
Raw coffee, not blended	7.2	7.1	7.2	+0.1
Raw coffee, blended	7.2	7.4	7.3	−0.1
Roasted coffee, T	...	3.6	3.2	−0.4
Roasted coffee, L	...	3.3	2.8	−0.5
Roasted coffee, K	...	3.2	2.6	−0.6
Roasted coffee, S	...	2.5	1.7	−0.8
Roasted coffee, Hag	...	3.5	3.2	−0.3

into consideration the dry matter of the extract and its composition. Were only the beverage concerned, determination of caffeine also might well be made only on the extract; the total content of the alkaloid, however, is of importance in the commercial extraction of caffeine from inferior grades or damaged beans.

McGill Hot Water Method.[38] Weigh into a counterbalanced flask a quantity of the finely ground sample equivalent to 10 g. of dry matter and add water up to 110 g. Heat to boiling in 10 to 15 minutes and continue boiling for 1 hour under a reflux condenser. Cool, filter through a dry paper, and use the extract for the determination of specific gravity and proximate constituents.

McGill had specially in mind the detection of chicory, the extract of which has an average specific gravity at 15° of 1.02821 as compared with that of coffee with an average of 1.00986. On these figures he based the formula for calculating the percentage of chicory (C) from the specific gravity (U) of the extract of the unknown:

$$C = \frac{(1.02821 - U) \times 100}{1.02821 - 1.00986}$$

The examination of the 10% extract has been extended by Lythgoe[39] to include, in addition to the specific gravity at 15° (1.0101 to 1.0113), the immersion refractometer reading at 20° (25.5 to 29.6), refractive index at 20° (1.33724 to 1.33804), solids (2.46 to 2.77), and ash (0.30 to 0.40).

Although in domestic practice boiling is not continued for 1 hour, it is not too long for complete extraction, which is essential for concordant results.

3. CHOCOLATE AND COCOA

The cacao or cocoa bean is not a true bean, nor does it closely resemble a bean. As a result of fermentation and roasting it is dark colored. *Plain chocolate* consists of shelled cocoa beans (cocoa nibs) ground to a smooth paste which on cooling forms a hard cake stiffened by the solidified fat. *Cocoa* is chocolate from which about half of the fat has been expressed. *Sweet chocolate* contains added sugar and flavor, which in the United States is commonly vanilla. *Milk chocolate* is sweet chocolate with which has been incorporated milk powder.

Microscopic Structure. See first page of Part II, I.

Chemical Composition of Chocolate and Cocoa. Characteristic of chocolate are

the purines, theobromine and caffeine, and the high percentage of fat or cacao butter. Less than 10% of starch is present. The content of fat in cocoa differs according to the amount originally present and the amount removed.

The general composition of plain and sweet chocolate and of cocoa is shown in the following table:[40]

Average Composition of Chocolate and Cocoa

	Water	Protein *	Theobromine	Caffeine	Fat	Sugar	Crude Starch	Pure Starch	Nifext †	Fiber	Ash
Chocolate											
Plain	3.78	16.64	0.78	0.13	52.19		11.63	8.11	16.64	2.86	3.15
Sweet	2.17	4.58	0.35	0.08	23.51	56.44	4.16	2.88	7.64	0.96	1.40
Cocoa	6.23	18.34	1.15	0.16	26.69		15.81	11.14	26.32	4.48	5.49

* Total nitrogen, less nitrogen of theobromine and caffeine, times 6.25. † Other than starch and sugar.

Unlike the two other common beverage alkaloidals, chocolate and cocoa are rich in nutrients and are consumed in their entirety.

a. Unsweetened Chocolate and Cocoa

Sample

Chill a whole cake of chocolate and a fine grater by storing in a refrigerator for some hours. Reduce the whole cake to a fine powder in a cool room by gentle grating; store in a cool place, and weigh out the portion for analysis while still cool. Cocoa, unless caked, needs only to be well mixed.

Water

Hydrogen Drying Method. See General Methods Part I, C2*a*. This method is theoretically exact, but for practical purposes drying in an open dish is satisfactory. Melted chocolate and cocoa rich in fat form a solid mass on cooling that repels moisture.

Protein

Calculation Method. Determine total nitrogen by the Kjeldahl-Wilfarth or the Kjeldahl-Gunning method. The addition of copper sulfate, as in the Gunning-Arnold method, is not required for the complete conversion of the nitrogen of theobromine and caffeine into ammonia.

By means of the factors 0.311 and 0.264, calculate from the percentages of theobromine and caffeine respectively the percentages of nitrogen in these forms and deduct the sum of these from the percentage of total nitrogen. Multiply the remainder by 6.25 to obtain the percentage of protein or, more exactly stated, nitrogenous matter other than alkaloids.

Caffeine and Theobromine

Decker Chloroform-Benzene Extraction Method for Caffeine.[41] After a study of the available methods, the Decker method was employed by Winton, Silverman, and Bailey [42] in a series of analyses of cocoa beans, chocolate, and cocoa from various sources. It permits the subsequent determination of theobromine in the same weighed portion, both alkaloids being obtained in the crystalline form with practically theoretical melting points.

Process. *Magnesia Defecation.* Boil for

30 minutes 10 g. of chocolate or cocoa or finely ground cocoa nibs or shells with 5 g. of *calcined magnesia* and 300 ml. of water. Filter on a disk of paper fitted to a Büchner funnel and transfer the paper and residue to the original flask. Add 150 ml. of water, boil for 15 minutes, and filter. Again transfer the paper and residue to the original flask and repeat the boiling with 150 ml. of water and filtration, but reject the residue after washing once or twice with hot water. Transfer the united filtrates to a Hoffmeister *Schälchen* (adding *quartz sand* if sugar is present) and evaporate to dryness.

Chloroform Extraction. Grind the dish and contents to a coarse powder in a mortar provided with a guard against flying, transfer to the inner tube of a suitable continuous-flow extractor, and subject to hot extraction with *chloroform* for 3 hours, or until the alkaloids are removed, into a weighed flask. Distil off the chloroform and dry the residue at 100° to constant weight.

Chocolate, cocoa, and cocoa nibs yield an extract consisting of practically pure theobromine and caffeine, but the extract of cocoa shells is less pure as indicated by the color.

Separation of Caffeine from Theobromine. Add to the extract in the flask 50 ml. of *benzene*, allow to stand for some hours with occasional shaking, then filter into a tared dish through a small paper, wash with *benzene*, and evaporate the solvent from the filtrate, dry to constant weight in a boiling water oven, and weigh, thus obtaining the weight of caffeine.

Kunze Silver Nitrate Volumetric Method for Theobromine.[43] PROCESS. *Silver Theobromate Precipitation.* Add to the residue and paper from the titration of the benzene solution above 150 ml. of water and *ammonium hydroxide* to slight alkaline reaction, then from a buret add an excess of *0.1 N silver nitrate solution.* Boil down to half the original volume, add 75 ml. of water, and repeat the boiling. If the excess of ammonium hydroxide is not removed, repeat the addition of water and boiling. Collect the precipitated silver theobromate on a paper and wash with hot water.

Titration. Determine the excess of silver nitrate in the filtrate by Volhard's method as follows.

Add 5 ml. of cold *saturated ferric ammonium alum solution* and enough *boiled nitric acid* to bleach the liquid. Titrate with *0.1 N ammonium thiocyanate solution* to the appearance of a permanent red color.

CALCULATION. Use the equation: 1 ml. of 0.1 *N* silver nitrate solution = 0.01802 g. of theobromine.

NOTE. If the mixed alkaloids, obtained as described under Caffeine above, are colorless, the weight of theobromine obtained by subtracting the weight of caffeine from that of the total alkaloids will usually agree closely with that by the silver nitrate titration.

Ceriotti Acid Digestion Benzene-Chloroform Extraction Gravimetric Method.[44] PROCESS. *Acid Digestion.* Defat 10 g. of the sample and reflux the residue for at least 1 hour with 150 ml. of *5% sulfuric acid.* Precipitate tannins, resins, and colors by addition of 200 ml. of *3% mercuric acetate solution*, and reflux for 10 minutes longer. Filter, wash, neutralize the filtrate with *calcined magnesium oxide*, and evaporate to 50 ml. on a water bath. Add 10 g. of *sand* and an excess of *calcined magnesium oxide*, and evaporate to full dryness.

Alkaloids Extraction. Extract the residue in a Johnson or Soxhlet extractor with *1 + 1 benzene-chloroform mixture*, transfer the extract to a tared dish, evaporate, dry, and weigh the mixed alkaloids.

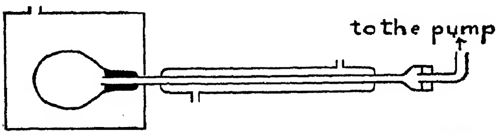
Caffeine Extraction. Remove the caffeine by three successive treatments with *carbon tetrachloride*, dry the residue of theobromine, and weigh.

Evaporate the carbon tetrachloride solution of caffeine and weigh. Also obtain the weight of caffeine by difference.

Cappelli Alkali Digestion Chloroform Extraction Gravimetric Method for Theobromine.[45] The method is based on the solubility of theobromine and the insolubility of caffeine in alkaline solution. The isolation of the joint alkaloids is effected by a procedure closely resembling that of the Decker method and the removal of the caffeine is by benzene as in the Kunze method or by carbon tetrachloride, as in the Ceriotti method.

APPARATUS. *Assembly* for the extraction of lighter with heavier liquid. See Introduction, Apparatus.

PROCESS. *Alkali Digestion.* Add to 50 ml. of cold *0.5 N sodium hydroxide* 10 g. of the sample from which the fat has previously been extracted by *naphtha.* Mix thoroughly, make up to 300 ml. in a volumetric flask, and again shake until homogeneous, then allow to stand 24 hours.



Courtesy of Chem.-Anal. 1940, **29,** *38*

FIG. 187. Wachs Theobromine Sublimation Assembly.

Chloroform Extraction. Remove 150 ml. of the clear supernatant liquid (equivalent to 5 g. of the sample) to the chamber of an assembly for extraction of lighter with heavier liquids, together with 50 ml. of *anhydrous chloroform* and 12 to 13 ml. of *normal sulfuric acid* until slightly acid to *litmus.* Place in the extraction flask 75 ml. additional of *chloroform* and extract for 6 hours, then evaporate the chloroform in a current of dry air, thus obtaining the theobromine contaminated with only traces of caffeine.

Caffeine Removal. Treat the residue with cold *benzene,* as in the Decker method, or with *carbon tetrachloride,* dry the theobromine at 100 to 105°, and weigh.

CALCULATION. Multiply the weight of the theobromine by 20 to obtain the percentage in the fat-free sample.

Wachs Sublimation Method for Theobromine.[46] The procedure was devised at the laboratory of the Dodge and Olcott Co. in view of the large quantity of solvent required in an extraction method and the impurity of the extract.

APPARATUS. The *Assembly* (Fig. 187) consists of a small flask, a condenser with a 15-cm. tube joined to the flask by a glass joint, an air bath, and a vacuum pump.

PROCESS. Weigh the flask and condenser tube, introduce into the flask a suitable amount of the sample, and weigh again. Connect the flask to the condenser tube and the latter to the vacuum pump. Heat the flask in the air bath to about 150° at such a rate that the crystalline theobromine collects in the condenser tube. At the end of the operation, weigh the flask and condenser tube. The increase in weight of the condenser tube is the weight of the theobromine, the loss in weight of the flask plus the charge serves as a rough control, allowing for water in the sample.

FAT

Ether Dry Extraction Method. Weigh 3 g. of the sample into the inner tube of a Johnson, Knorr, or similar extractor and dry for at least 24 hours in a sulfuric acid desiccator. Extract with *anhydrous ether* until no more fat is removed. Since the ether runs through the material at an exceedingly slow rate, it is advisable to interrupt the heating from time to time to allow the tube to empty. Slowness, however, means efficiency and the filling and emptying of the tube with three portions of approximately 10 ml. each of *ether* is usually sufficient.

Stanley Refractometric Method.[47] The method as developed at the W. A. Cleary Corporation, New Brunswick, N. J., is as follows.

APPARATUS. *Abbé Refractometer* or *Butyrorefractometer.*

A. PROCESS FOR ABBÉ INSTRUMENT. *Filtration.* Weigh 20 g. of the sample and 20 g. of *tricresyl phosphate* into a 100-ml. low-form beaker. Keep in a warm place (as high as 75° for chocolate, but not over 40° for milk chocolate) until the chocolate has melted, then thoroughly stir and return to the warm place. After 10 minutes stir and filter (without a funnel) on a 24-cm. No. 1 Whatman pleated paper or its equivalent, resting in a tall-form lipless Pyrex beaker so that the tip touches the bottom. Keep in a warm place during the filtration and collect 5 to 10 ml. of filtrate.

Refractometer Reading. Take the reading at 35 to 45° in an Abbé refractometer with temperature controlled thermostatically at 40°.

Blank. To obtain the zero point for the solvent, run a blank determination as above directed, but omitting the fat mixture.

CALCULATION. The difference between the reading of the blank and its supposed reading (1.546 to 1.550 on the graph), seldom over a few units in the fourth place of decimals, constitutes the correction due partly to slight differences in the composition of the solvent or a slight shift in the zero point of the instrument. Find the percentage of cacao butter corresponding to the refraction in one of three graphs published in the *Journal* or obtainable in larger form from the American Lecithin Co., Elmhurst, N. Y.

B. PROCESS FOR BUTYROREFRACTOMETER. Use *dibutyl phthalate* as the solvent and only half the weight directed for *tricresyl phosphate*, digest at a temperature not to exceed 50°, read the refraction at 48°, and consult two special graphs given in the *Journal* to obtain the percentage of fat.

FOREIGN FAT

The substitution of cheaper fats for a portion of the cacao butter is detected by the systematic determination of the fat values beginning with melting point. High Reichert-Meissl and Polenske numbers indicate the presence of cocoanut and palm kernel oils in the absence of milk fat. The silver number is also useful in detecting these oils. Of particular value in detecting various foreign fats are the critical temperature of dissolution of fat in acetic acid and the acetone-carbon tetrachloride test.

Valenta Acetic Acid Turbidity Method.[48] Of methods for determining the critical temperature of dissolution (turbidity) of the fat discussed in Part II, B, the Valenta method, as studied by Bloomberg,[49] by Baughman,[50] and others, has proved reliable in detecting foreign oils.

APPARATUS. A 15 x 2 cm. (6 x 0.75 inch) test tube fitted with a cork carrying a thermometer reading to 0.1° that dips below 10 ml. of liquid. The test tube is enclosed in a second tube, 10 x 3 cm. (4 x 1.25 inch) and is held in place by a cork with a groove for equalizing pressure.

PROCESS. Filter through a dry paper in an oven heated to 110° to remove moisture. While it is still barely warm, weigh 5 g. of the fat and 5 g. of *99.5% acetic acid* into the inner test tube and insert the cork and thermometer. Heat with frequent shaking in a glycerol bath until the fat and acid form a clear liquid. Allow to cool with continued shaking without removal from the bath and note the temperature of the first appearance of turbidity. Conduct a test in like manner on pure cacao butter.

Determine the acid number (milligrams of potassium hydroxide required to neutralize the free fatty acids in 1 g. of fat) of the sample and pure cacao butter, then multiply the number by a factor which is 1.4° if the turbidity temperature of the pure cacao butter is 90°, decreasing proportionally to 1.2° if the turbidity temperature is 100°, and add the correction thus obtained to the reading.

If, after the correction is added, the tur-

bidity temperature of the fat from the sample is more than 3° lower than that of chocolate or sweet chocolate, or more than 6° lower than that of milk chocolate, adulteration with cocoanut, palm kernel, corn, peanut, cottonseed, etc., oils or their stearins is indicated.

Bloomberg Acetone-Carbon Tetrachloride Method.[51] PROCESS. Filter the warm sample in an oven at 110°. Dissolve 5 g. of the dry warm filtered fat in a test tube in 5 ml. of *1 + 1 acetone-carbon tetrachloride mixture*. Cool in ice water 20 to 30 minutes. Treat 5 ml. of pure cacao butter in the same manner.

If hydrogenated oil, tallow, oleostearin, or paraffin is present, a white flocculent precipitate soon forms and, if the water is sufficiently cold, cacao butter may solidify. If the precipitate forms, remove the test tube from the ice water and allow to stand at room temperature for a time. Solidified cacao butter soon will melt and go into solution, but a precipitate due to any of the adulterants used requires a longer time.

Paschke Lower Acids Saponification Method.[52] Since the only acids present in the glycerides of cacao butter are palmitic, stearic, and oleic, any fats containing lower acids are detected by the isolation of these acids, determination of their saponification numbers, and calculation (Wurzburg University).

PROCESS. *Esterification.* Reflux briskly for 5 to 6 hours 21 g. of the fat with 150 ml. of *ethanol* and 3 ml. of *sulfuric acid*.

Naphtha Extraction. Pour the solution into 1 liter of water and extract the separated esters with *naphtha-ether mixture*. Wash the extract several times with an equal volume of water and dry over *anhydrous sodium sulfate*. Remove the solvent by distillation and drying on a water bath for 2 hours.

Fractionation. Distil the esters *in vacuo* until 50% has passed over, then redistil until 3 to 3.3 g. are obtained.

Saponification. Using 2.5 g. of each distillate and 30 ml. of *0.5 N ethanolic potash*, demine the saponification number of the first distillate (S_1), the second distillate (S_2), and the residue (S_3) and calculate the weight of foreign fat (F) by one of the following formulas, the first being the more accurate:

$$F = S_2 - S_1 - 5.5$$

or

$$S_2 - S_3 - 9.5$$

SILVER NUMBER

Wijsmann and Reijst Volumetric Method.[53] Cocoanut oil and palm kernel oil are richer in caproic and caprylic acids than butter fat and cacao butter. This difference is measured by the silver number which, although adversely criticised by German authors, in modified form is of distinct value.

Bertram, Bos, and Verhagen Magnesium Sulfate Modification.[54] The following details, elaborated by Winkler [55] and others, are substantially as tentatively adopted by the A.O.A.C.

PROCESS. *Saponification.* Heat on a steam bath 10 g. of the fat with 40 ml. of *ethanol* and 5 ml. of *75% potassium hydroxide solution* until saponified. Evaporate to dryness, take up the soap in 150 ml. of water, cool, and dilute to 250 ml. in a volumetric flask.

Magnesium Sulfate Treatment. Pipet 200 ml. of the soap solution into a 500-ml. Erlenmeyer flask, stopper with a cork carrying a thermometer, and heat to 80° in a water bath. Pipet into the flask 50 ml. of *15% magnesium sulfate solution*, rotate, replace the stopper and thermometer, and heat for 8 to 10 minutes at 70 to 80° with occasional rotation. Cool to 20 to 25° under a tap with shaking, stopper with a solid cork, and shake vigorously for 4 minutes. Maintain at 20 to 25° until a water layer separates at the bot-

tom. Filter through a Büchner funnel while pressing the mass with a horn spoon.

Silver Precipitation. Pipet 200 ml. of the filtrate into a 250-ml. volumetric flask and neutralize to *phenolphthalein* with *0.5 N sulfuric acid.* Dissolve in the solution 20 g. of *crystalline sodium nitrate* and add 22.5 ml. of *0.2 N silver nitrate solution.* Dilute to volume, shake 3 minutes, let stand a short time, and filter through a dry pleated paper.

Titration. Add to 200 ml. of the filtrate 6 ml. of *saturated ferric (or ammonium) sulfate solution* and 4 ml. of *40% nitric acid,* then titrate with *0.1 N ammonium thiocyanate solution* to the first color change.

CALCULATION. Obtain the silver number (A) by the formula

$$A = 2.107(N - S)$$

in which

$$2.107 = \frac{10.788}{5.12}$$

$$\frac{\text{mg. of Ag per } 0.1\,N}{\text{g. of fat in aliquot titrated}}$$

$N = 1.6 \times$ ml. of $0.2\,N$ $AgNO_3$ added, and S = ml. of $0.1\,N\,NH_4SCN$ solution used in back-titration.

Make a determination on 10 g. of cacao butter for comparison.

EXAMPLES. Silver numbers of palm kernel and cocoanut oils range from about 26 for the stearins to 60 for whole cocoanut oil. For butter fat the number is about 11.6 and for cacao butter 0.6.

PECTIN

(Pectic Acid)

Winkler Oxalate Extraction Acid Ethanol Precipitation Gravimetric Method.[56] The method was devised at the laboratory of the U. S. Food and Drug Administration for the purpose of detecting cocoa shells in chocolate, milk chocolate, and cocoa.

PROCESS. *Extraction.* To 5 g. of fat-free liquor (chocolate or cocoa) or 16 g. of fat-free sweet chocolate in a beaker, add 10 ml. of *0.5% ammonium oxalate solution,* make into a paste, then add 200 ml. additional and mix. Cover, heat on a steam bath for 3 to 3.5 hours with occasional stirring, cool, again stir, and transfer to a centrifuge bottle, using water and a policeman. Add 2.25 ml. of *glacial acetic acid,* stopper, shake, and let stand 5 minutes, then centrifuge at 1800 to 2000 r.p.m. for 15 to 20 minutes and decant the liquid back into the beaker. If plain chocolate, add 4 g. of sugar. Repeat the treatment, using, however, 100 ml. of *oxalate* and 1 ml. of *acid.*

Tannin Precipitation. Boil down the joint extracts to 125 ml., cool, add 10 ml. of *10% tannic acid (tannin) solution,* let stand 10 minutes, stir, transfer to a centrifuge bottle, using a little water, whirl for 20 minutes, and decant if clear into the original beaker. If not clear, decant into another bottle, add 10 ml. more of *tannic acid,* whirl for 15 minutes, and again decant.

Acid-Ethanol Precipitation. Evaporate the liquid to 50 ml. (not below 40 ml.) and precipitate the pectin with a mixture of 250 ml. of *ethanol* and 3 ml. of *1 + 1 hydrochloric acid.* After allowing to stand covered for 30 minutes, collect the precipitate on a pleated paper and wash with *ethanol.*

Reprecipitation. Wash the precipitate back into the beaker with hot water, heat to boiling, filter if a precipitate forms, adjust to about 90 ml. at room temperature, and cool below 25°. Add 10 ml. of *10% sodium hydroxide solution,* mix, let stand 20 minutes, dilute to 400 ml., add 3.5 ml. of *glacial acetic acid,* then mix and add 10 ml. of *molar calcium chloride solution.* Stir, let stand 5 minutes, and boil a few minutes. Filter on a pleated paper, wash with hot water, rinse the precipitate back into the beaker with hot water, and adjust to 100 ml. Finally add 3.5 ml. of *1 + 1 hydrochloric acid,* boil gently for

5 minutes, filter, wash with hot water, rinse the pectic acid from the paper to a platinum dish, evaporate to dryness, dry at 100°, and weigh. Ignite, reweigh, and report the loss as pectic acid.

NOTE. In his second paper, Winkler introduces slight changes. The tannin precipitation is carried out at 8 to 10°, the residue after extracting the pectin is dried and weighed, the fat-free cocoa mass obtained with a factor, the calcium precipitation is deleted, and ethanol extraction follows the ether extraction. Charges are weighed and the extracting solution is increased from 210 to 350 ml. A procedure is also given for milk chocolate.

EXAMPLES. Pectin in liquor 0.14, in chocolate containing 9.45% of added shells 0.65, and in cocoa shells 4.8%, dry, sugar-, and fat-free basis.

SUGARS

See Sweet Chocolate and Sweet Cocoa below.

CRUDE STARCH

Sachsse Direct Acid Hydrolysis Copper Reduction Gravimetric Method. As modified for cocoa products, the procedure is as follows.[57]

REAGENT. *Lead Subacetate Solution.* Boil for 30 minutes 430 g. of normal lead acetate and 130 g. of PbO with 1 liter of water, filter, and dilute to 1.25 sp.gr.

PROCESS. *Fat Extraction.* Remove the fat from 4 g. of the sample either by grinding with successive portions of *ether* or *naphtha*, decanting, and filtering on a hardened paper after each, or by shaking with the solvent, centrifuging, decanting, and filtering.

Sugar Extraction. Remove sugar or sugars in the manner directed for fat extraction, substituting cold water, or preferably *10% ethanol*, for the fat solvent and filtering on the paper previously used. Sweet chocolate and cocoa require especially thorough washing, using at least 500 ml. of water, although the determination of sugar is made on a separate portion.

Acid Hydrolysis. Transfer the wet residue from the paper to a 500-ml. flask, using 200 ml. of water delivered from a small wash bottle and gently rubbing the paper so as to remove the last traces of starch. Add 20 ml. of *25% hydrochloric acid* and heat for 3 hours in a boiling water bath.

Clarification. Cool, nearly neutralize with *10% sodium hydroxide solution,* clear with 5 ml. of *lead subacetate solution,* make up to 250 ml. in a volumetric flask, and filter through a dry paper.

Deleading. Remove the lead from 100 ml. of the filtrate with *powdered anhydrous potassium oxalate* and filter.

Copper Reduction. Determine the copper reducing power in 50 ml. (= 0.8 g. of the sample) by the Munson and Walker or the Allihn Method, as given in Part I, C6*a*, and calculate the starch from the dextrose by the factor 0.9.

Märcker Diastase Extraction Copper Reduction Gravimetric Method.[58] REAGENTS. *Malt Extract.* Digest for 2.5 hours 100 g. of powdered fresh malt with 1 liter of water and filter, returning the first portions to the paper until the filtrate runs clear.

Alumina Cream. Precipitate a standard solution of $Al_2(SO_4)_3 \cdot 18H_2O$ with an excess of NH_4OH, wash repeatedly by decantation until all sulfate is removed, then store as an emulsion.

PROCESS. *Preliminary Treatment.* Remove the fat and, if present, the sugars as in the direct acid hydrolysis method. Carefully wash the wet residue from the hardened paper into a beaker with 100 ml. of water, rubbing the paper with your finger tip to aid in removal of the starch. Heat on an asbestos plate to gentle boiling with continual stirring and boil for 30 minutes. Replace the

water lost by evaporation and immerse in a water bath at 55 to 60°.

Diastase Digestion. When the solution acquires the temperature of the bath, add 10 ml. of fresh *malt extract* and digest for 2 hours with occasional stirring. Repeat the boiling, cooling, and digestion with another 10-ml. portion of *malt extract*, then heat to boiling to kill the enzyme, cool, and transfer to a 250-ml. volumetric flask.

Clarification. Add 3 ml. of *alumina cream* to insure a clear filtrate, make up to the mark, mix, and filter through a dry paper.

Copper Reduction. Pipet 200 ml. of the filtrate into a 500-ml. flask. Conduct the acid hydrolysis and copper reduction as directed above for the Sachsse Direct Hydrolysis Method.

Fiber

Use the residue from the determination of fat or extract a fresh charge and proceed as in the following method.

Official A.O.A.C. Method for Cacao Products other than Milk Chocolate.[59] Process. Treat 7 g. of liquor or 50 g. of sweet chocolate with at least two 100-ml. portions of *ether*, decanting after each. Dry at 100° and grind the residue. Shake and centrifuge three times with 100-ml. portions of water, then twice with 100 ml. of *ethanol*, and then once with 100 ml. of *ether*. Dry the residue to constant weight, grind, and determine fiber in 2 g. by the usual method, using, however, linen for both filtrations and calculating the result as percentage of the sample.

Calculation. Multiply the percentage of fiber by 0.7 to obtain the percentage on the water-, fat-, and sugar-free basis.

Ash

Determine total ash, water-soluble and water-insoluble ash, alkalinity of water-soluble and water-insoluble ash, and acid-insoluble ash (sand) as directed under General Methods (Part I, C2*f*).

Color

(Cocoa Catechin and Cocoa Tannin)

Adam Cinchonine Sulfate Gravimetric Method.[60] According to Adam (Bristol), who elaborated the methods of Mitchell [61] and Miss Price [62] and the modification of Glasstone,[63] the red and brown colors are complex alteration products of catechin and tannin originally present in the unfermented cacao bean. He divides them into (*a*) catechin-like substance (cocoa catechin), (*b*) catechutannin (cocoa tannin), and (*c*) a compound of cocoa catechin plus caffeine.

According to Price, catechin is

$$C_{15}H_9O(OH)_5 \cdot 3H_2O$$

Apparatus. *Colorimeter.*

Reagent. *Mitchell Reagent.* Dissolve 0.1 g. of ferrous sulfate and 0.5 g. of Rochelle salts in 100 ml. of water. The solution must be freshly prepared.

Process. *Removal of Fat and Xanthine Bases.* Extract the finely divided sample with 150 g. of *naphtha* in a Soxhlet extractor, grind the residue with acid-treated *sand*, and continue the extraction for 2 days. Regrind a second time and extract with *chloroform* for 5 days.

A. Cocoa Catechin. Extract the residue from the chloroform extraction with *ether* for 6 days, evaporate the ether, take up in hot water, and make up to a definite volume. To 85 ml. of water in a 100-ml. colorimeter tube, add 1 ml. of the solution of the unknown, 8 ml. of *10% ammonium acetate*, and 2 ml. of the *Mitchell reagent*. Compare the color with standard solutions of *0.10 to 0.15% anhydrous crystalline catechin* treated in like manner.

B. Cocoa Tannin.[64] Dry the powder left after the removal of cocoa catechin and shake with a suitable volume of water at 60°, filter, and add an equal volume of *saturated cinchonine sulfate solution*. After coagulation of the precipitate, filter through a weighed

alundum crucible, wash with *dilute cinchonine sulfate solution*, dry in the air, then to constant weight in a steam oven.

As cocoa catechin precipitates with cinchonine sulfate only after prolonged boiling with weak acid or alkaline solution, tannin determinations may be carried out without its removal. The precipitate, however, may contain tannin-like alteration products of cocoa catechin formed by oxidation and condensation during fermentation.

Examples. Fermented cocoa nibs: catechin none; tannin 1.70 to 1.97%. Unfermented or poorly fermented cocoa nibs: catechin 0.62 to 0.80%; tannin 2.37 to 3.48%.

b. Sweet Chocolate and Sweet Cocoa

The analytical methods for sweet chocolate and sweet cocoa differ from those given under Chocolate and Cocoa chiefly in that (1) larger amounts (about double) are weighed out to allow for the dilution with sugar, (2) sucrose is extracted by more thorough treatment with water preliminary to the determination of constituents such as starch, theobromine, caffeine, and fiber, and (3) sucrose itself is determined by the polarimetric method. The removal of sugar must be complete in starch determination.

Sucrose

Determination of sucrose in sweetened cacao products is of importance in Customs' laboratories where duties on the sweetened product and the sugar are involved.

Seeker, Shanley, and Lourie Method. The formulas were developed at the laboratory of the U. S. Food Administration [65] and later employed by Fitelson [66] in connection with lactose determination.

Process. Shake 26 g. of the sample in a nursing bottle for 5 minutes with 100 ml. of *naphtha*, centrifuge, and decant. Repeat the treatment and keep the bottle in a warm place to expel the naphtha. Add exactly 100 ml. of water from a pipet, shake to detach most of the chocolate adhering to the bottle, loosen the stopper, and heat for 15 minutes in a water bath at 85° to 90° with occasional shaking. Cool and add from a pipet exactly 5 ml. each of *basic lead acetate solution* (sp.gr. 1.25) and water, shake, centrifuge, and filter through a dry paper. Precipitate the excess of lead with *powdered potassium oxalate* and filter through a dry paper. Mix equal volumes of the filtrate and water and polarize in a 200-mm. tube at 20° before and after inversion in the usual manner (Part II, E2). Multiply both readings by 2 to correct for the dilution.

Calculation. Obtain the percentage of sucrose (S) from the following formulas:

$$d = \frac{P - P'}{143.0 - t/2}$$

$$X = \frac{0.2244(P - 21d)}{1 - 0.00204(P -}$$

$$S = \frac{(P - P')(110 + X)}{143.0 - t/2}$$

in which P and P' are respectively the direct and invert polarization in a 200 mm. tube at 20°, t is the temperature, d is apparent sucrose by the Clerget formula, and X is the volume correction.

c. Sweet Milk Chocolate

The methods given under Chocolate and Cocoa apply to sweet milk chocolate, except that larger amounts are weighed out to allow for the dilution with sugar and milk powder and that sugars, as well as fat, are removed by washing preliminary to determination of starch (complete removal essential), theobromine, caffeine, and fiber.

Additional methods follow.

Milk Proteins

Baier and Neumann Sodium Oxalate Method.[67] The casein in the milk solids is extracted with weak *sodium oxalate solution*, first employed by Arthus, then 5 ml. of *5% uranium acetate solution* are added, followed by *30% acetic acid solution* until the casein precipitates. After adding 5 drops more of *acid*, centrifuging, washing with a *solution containing 5 g. of uranium acetate and 3 ml. of 30% acetic acid in 100 ml.*, nitrogen is determined in the precipitate. Casein is calculated by the factor 6.37 and milk proteins from the casein by the factor 1.111.

Winkler Oxalate Extraction Tannin Precipitation Nitrogen Factor Method.[68] Process. *Ether Extraction.* Defat 10 g. of the sample by shaking and centrifuging in a 250-ml. centrifuge bottle with two consecutive portions of 100 ml. of *ether*.

Oxalate Treatment. Remove the last trace of ether from the extracted residue, add from a pipet exactly 100 ml. of water, stopper, and shake vigorously for 4 minutes, then add 100 ml. of *1% sodium oxalate solution*, stopper, and shake vigorously for 3 minutes. Allow to stand 10 minutes and shake for 2 minutes, then centrifuge at 1800 r.p.m. for 15 minutes, and decant.

Acid Tannin Precipitation. Pipet 100 ml. of the clear solution into a 250-ml. beaker, add 1 ml. of *glacial acetic acid* while gently stirring, let stand a few minutes, then add 4 ml. of *freshly prepared 10% tannic acid solution* and stir. Allow the precipitate to separate and settle, then filter on a 589 white label paper, overlaid with paper pulp, in a 7-cm. Büchner funnel, and wash once or twice with *1% sodium oxalate solution*, to each 100 ml. of which 1 ml. of *glacial acetic* and 2 ml. of *10% tannic acid* have been added.

Nitrogen Determination. Digest in the usual manner, using, however, 20 ml. of *sulfuric acid*, 15 g. of *sodium sulfate*, and 1 g. of the *catalyst* (*1 g. of selenium to 5 g. of mercuric oxide*). Distil in the usual manner, using, however, 50 ml. of a solution containing 300 g. of *sodium hydroxide* and 10 g. of *sodium thiosulfate* in 500 ml. of water.

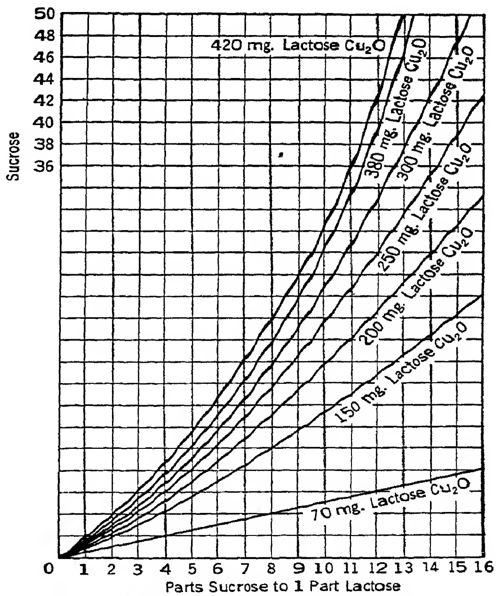
Calculation. Multiply the per cent of nitrogen by 6.38 to obtain the per cent of casein plus albumin, and the product by 1.07 to obtain the per cent of total milk protein.

Cocoanut Oil and Palm Kernel Oil

The presence of one of these foreign fats is indicated by a high Polenske number, but the range for the individual fats is so wide as to render calculation of the amount of foreign fat extremely inaccurate.

Lactose

Fitelson Polarimetric Copper Reduction Method for Lactose.[69] This tentative pro-



Courtesy of Methods of Analysis, A.O.A.C. 1935, p. 200

Fig. 188. Fitelson Lactose Correction Graph.

cedure for calculating lactose, developed at the U. S. Food Inspection Laboratory, New York, has displaced the Dubois method [70] in recent editions of the *A.O.A.C. Methods*, since it was believed that copper reduction, with suitable correction for the influence of sucrose, combined with polarization, is more accurate than the wholly polarimetric method and that thiosulfate titration yields better results than the gravimetric method.

PROCESS. After defecation with basic lead acetate solution polarize by the usual method, also, after deleading, determine the copper reduction by the Munson and Walker method in an aliquot (usually 20 ml. diluted to 50 ml.) of the solution obtained as directed under Sucrose above.

CALCULATION. Two formulas are employed, one for the approximate percentage (L') of lactose and one for the corrected percentage (L). A graph (Fig. 188) for the correction of the cuprous oxide, according to the ratio of sucrose to lactose and the weight of lactose, is also employed.

$$L' = \frac{P(1.1 + 0.01X) - S}{0.79}$$

$$L = \frac{L(110 + X)}{0.26V}$$

in which P is the direct polarization, S is the percentage of sucrose, X is the factor calculated by the Seeker, Shanley, and Lourie formula, and V is the volume (usually 20 ml.) of solution used for copper reduction above.

FIBER

PROCESS. Treat 50 g. of the sample as directed in the A.O.A.C. Method for Chocolate, but interpose between the last ether and the first water extraction, shaking, centrifuging, and decantation, using 1 portion of 100 ml. of *1% sodium oxalate solution.*

CALCULATION. Multiply the percentage of fiber in the residue by 0.7 to obtain the fiber on the fat-, sugar-, moisture-, and milk-free basis.

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J. FOOD FLAVORS

1. SPICES

Spices are natural products of pronounced odor and taste (usually due to essential oils) that serve for flavoring and, to some extent, for preserving foods, but in the quantities used do not supply appreciable amounts of nutrients. They are classed according to the part of the plant as flower, fruit, seed, bark, and rhizome spices, and according to the region of production as tropical or temperate. The tropical spices, with a few exceptions such as allspice, the fruit of a West Indian tree, and Jamaica ginger, are products of the East Indies used in a broad sense to include the Dutch Islands, Indo China, Malaya, southern India, and tropical East Africa together with neighboring islands. Included as temperate spices are the leaves of members of the mint family (mint, spearmint, sage, marjoram, savory, thyme, and rosemary) and the fruits of the parsley family (anise, caraway, cumin, coriander, dill, celery, and fennel). Vanilla (the fruit of an orchid), tonka bean (the seed of a legume), and also some other products whose flavoring principles are not essential oils are classed for convenience with spices.

Microscopic Structure (Figs. 189 to 200). Aside from botanical classification by species and plant part, spices may be grouped as (1) starchy or starch-free, (2) the presence or absence of stone cells, and (3) the presence or absence of epidermal hairs and glands (leaf spices). The starchy spices are (*a*) black pepper with small polygonal grains, (*b*) cinnamon, cassia, nutmeg, and allspice with medium rounded grains, and (*c*) ginger with large pear-shaped grains with eccentric hilum. Pepper contains many forms of stone cells, one layer consisting of beaker-shaped forms. Isodiametric and elongated stone cells occur in cinnamon and cassia, also cork cells if not scraped. The gut-like cells of the seeds of paprika and cayenne are yellow, contrasting with the red oil drops. Cloves, in addition to numerous floral parts, contain pollen grains. These are but a few avenues of approach.

Schemes classifying spices by structure have been proposed but are of little use in the examination of mixtures. One must know well each spice, largely from a study of standard samples.

Adulteration. Before the passage of pure food laws, ground spices were grossly adulterated with materials with no flavoring value. Makeweights identified by microscopic examination were ground cocoanut shells roasted to imitate allspice, cloves, and black pepper; sawdust dyed to imitate cayenne pepper; flour and meal colored to imitate mustard, cinnamon, and ginger; and oil seed meal, ground peas, crushed biscuit, ground nut shells, and weed seed meal (from screenings matching various ground spices). Although these frauds are now rare in the United States, abandonment of microscopic examination would invite their return; furthermore the examination detects impurities indicative of low grade such as an excess of pepper shells in black pepper, stems, calyxes, and seeds in paprika, stems in leaf spices, China and Batavia cassia in the more valuable Saigon cassia, charlock in mustard flour, and undecorticated African ginger in Jamaica ginger. The technique is essentially as given at the beginning of sections A, B, C, and D of Part II.

Black pepper and ginger are distinctly starchy, cassia (cinnamon), allspice, nutmeg, and mace contain starch or its equivalent as a minor constituent, and cloves are starch-free. The magnification is ×160.

FIG. 189. Black Pepper. Elements in surface view. *t* bract hair. Fruit coat: *epi* epicarp with stoma, *hy* hypoderm with stone cells, *mes* mesocarp (above, left, with resin; right, with oil drops; below, porous cells), *st* stone cells, and end^1 endocarp (end^2 in cross section). Seed coat: *aep* outer epiderm, *m* middle layer, and *iep* inner epiderm. Perisperm: *ep* epiderm and *am* starch cells.

Minute starch grains make up the greater part of the kernel. Stone cells, some of beaker shape (end^2), *distinguish black from white pepper.*

FIG. 190. Allspice. Above, elements in surface view; below, in cross section. Fruit coat: *epi* epicarp with hairs and stoma. Seed coat: *aep* outer epiderm, *sub* subepiderm, and *br* brown cells; *F* fruit coat with rosette crystals, *ol* oil cavity, and *st* stone cells. *R* radicle with oil cavity (dark) and *am* starch grains.

The epicarp with short, thick-walled hairs, the large stone cells of the fruit coat, the brown cells, and the oil cavities are the characteristic fruit tissues. Starch cells and oil cavities form the bulk of the seed.

FIG. 191. Ginger. Elements of rhizome in surface view. *su* cork cells, *am* starch cells, *f* fibers, *v* vessels, *pig* pigment cell, and *ol* oleoresin cell.

The chief elements are the cork cells, the blunt-pointed starch grains, the reticulated vessels, and the broad fibers; all are conspicuous.

FIG. 192. China Cassia. Elements of powder. *su* cork layers in section: *a* arch cells, *sc* stone cells, and *tc* thin cells of cork layer; *m* medullary ray with starch grains, crystals, and thick-walled secretion cell; *f* bast fiber; *st* stone cells; *am* starch cells; *E* endosperm.

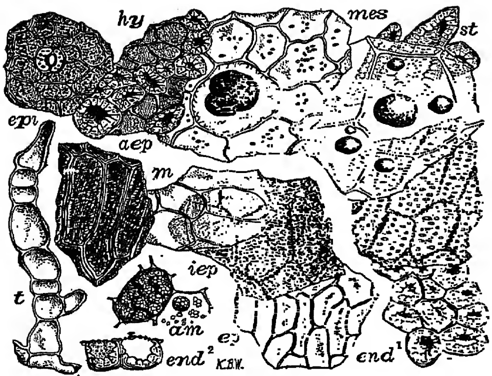
Cork cells with dark contents, large thick-walled fibers, large stone cells, and round starch grains of medium size are readily found in the powder.

FIG. 193. Mace and Nutmeg. Upper left, mace: epi^2 epiderm in surface view; epi^1 epiderm, *ol* oleoresin cell, and *ads* amylodextrin-starch cell in cross section. Right, nutmeg in cross section: *PN* cortex with crystals and *ca* calcium carbonate coating; *SN* secondary perisperm with *ol* oleoresin cells; *E* endosperm with *am* starch grains and *cr* crystals.

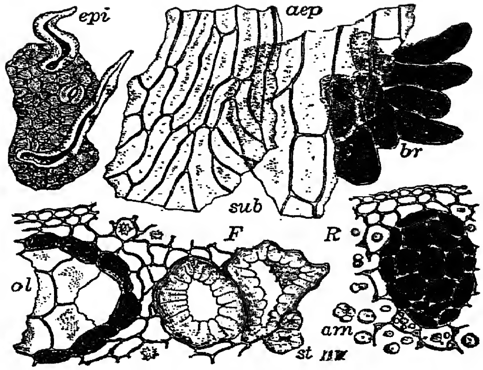
Mace is characterized by the elongated epidermal cells, the oil cavities and the unique amylodextrin-starch grains. In nutmeg, after ether extraction, medium-sized starch grains and monoclinic oxalate crystals are evident.

FIG. 194. Cloves. Elements of powder. Receptacle: *epi* epiderm with stoma, *b* chain cells, *f* bast fibers, *ol* volatile oil cavities, and *fv* fibrovascular bundle elements. *pn* anther with pollen grains. Corolla: *epc* epiderm and *r* reticulated cells. Stems: *st* stone cells. *m* pith.

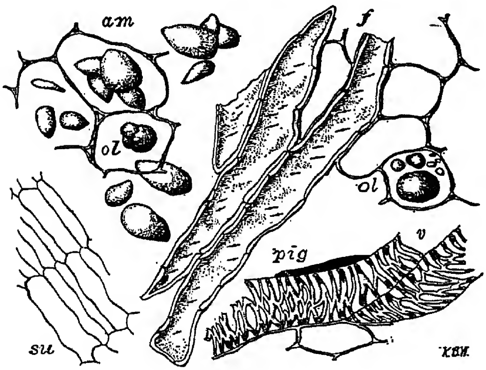
Of the great variety of tissues, the pollen grains, long thick-walled fibers, and the stone cells are seen after extraction of the volatile oil



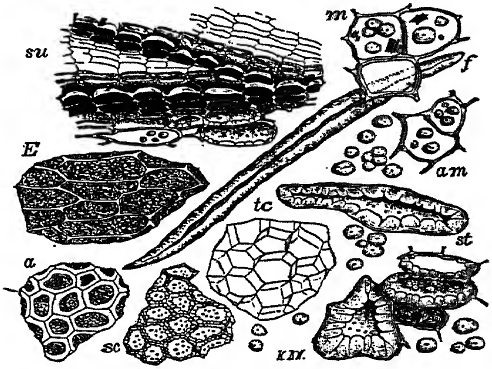
Black Pepper



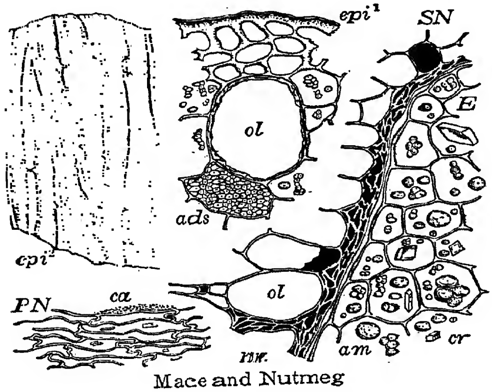
Allspice



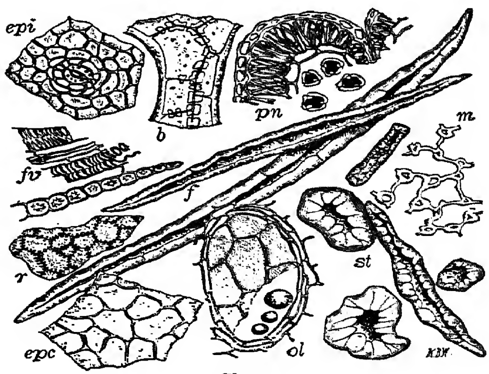
Ginger



China Cassia



Mace and Nutmeg



Cloves

FIGS. 189–194.

LEGENDS OF ILLUSTRATIONS ON FACING PAGE

White and black mustard are starch-free oily seeds, cayenne, paprika, and caraway are starch-free oily fruits, and sage is a leaf spice with no visible starch. The magnification is ×160.

Fig. 195. White Mustard. Upper left, seed in cross section; right, elements in surface view. Seed coat: *ep* outer epiderm, *col* collenchyma-like subepiderm, *pal* palisade cells (pal^1 outer and pal^2 inner portion), and *p* parenchyma. *E* endosperm. *C* outer epiderm of cotyledon.

The light color of the palisade cells and the presence of subepidermal cells with thickened corners (collenchyma) are characteristic.

Fig. 196. Black Mustard. Upper left, seed in cross section; right, elements in surface view. Seed coat: *ep* outer epiderm, *sub* subepiderm, *pal* palisade cells (pal^1 outer and pal^2 inner portion), and *pig* pigment cells. *E* endosperm. *C* outer epiderm of cotyledon.

The palisade cells are much darker than in white mustard, collenchyma is absent, and the pigment layer replaces the colorless layer. The reticulations, evident under a lens as well as with the microscope, are marked, but not so distinct as in brown mustard (not shown), and the meshes are smaller.

Charlock (not shown) responds to the chloral hydrate test.

Fig. 197. Cayenne. Left, fruit coat in cross section: epi^1 epicarp, *mes* mesocarp with oil drops, *fv* fibrovascular bundle, *g* giant cells, and *end* endocarp. Right, epi^2 epicarp and *S* outer epiderm of seed coat in surface view.

Starch is absent; orange-red oil drops are present. The epicarp cells approach the rectangular in form, whereas in paprika they are polygonal. In both spices, the epiderm of the seed coat has gut-like convolutions.

Fig. 198. Paprika. Elements in surface view. Calyx: *ae* outer epiderm with stoma and blunt hair, *cr* mesophyl with crystal sand, and *ie* inner epiderm with capitate hair. Fruit coat: *epi* epicarp, *mes* mesocarp with oil drops, and *end* endocarp. *S* outer epiderm of seed coat.

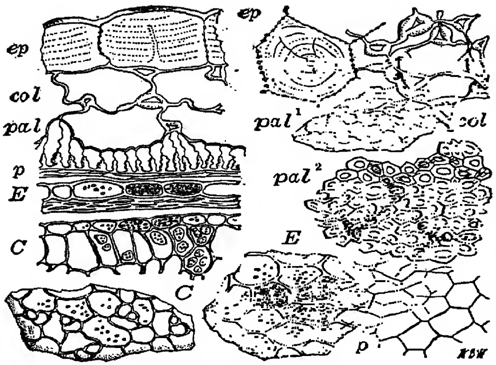
Certain distinctions from cayenne are noted above, others are brought out in the illustrations.

Fig. 199. Caraway. Elements of fruit in surface view. Fruit coat: *epi* epicarp with stoma, v^1 vitta at apex, v^2 at base, *fv* fibro-vascular bundle elements, end^1 endocarp at apex, and end^2 at base. Endosperm: *al* aleurone cells.

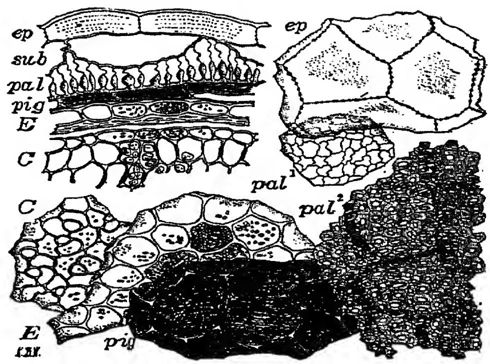
Typical of the umbelliferous group are the cells containing volatile oil (vittae); these are variously differentiated in anise, dill, fennel, coriander, cumin, and celery.

Fig. 200. Sage. Lower epiderm of leaf in surface view: *I* between veins with pointed and capitate hairs, stomata, and bladder gland; *II* over large vein with warty, thick-walled, jointed hairs.

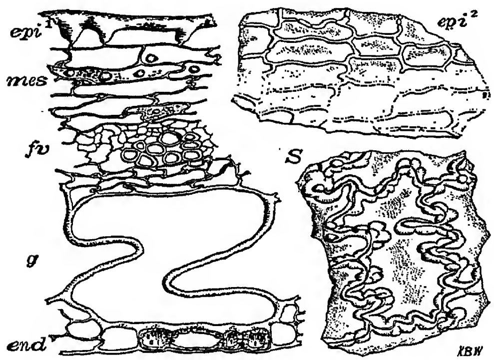
Epidermal hairs and volatile oil glands characterize the mint group. In sage, the hairs are numerous, variously differentiated, and matted. The number of cells in the bladder glands varies with the species (peppermint, spearmint, rosemary, sweet basil, sweet marjoram, thyme, and summer savory).



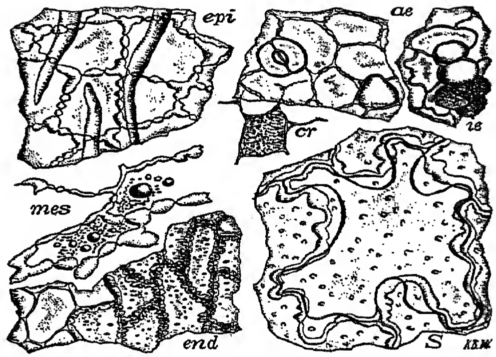
White Mustard



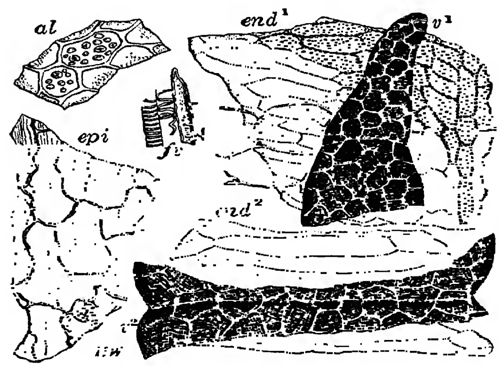
Black Mustard



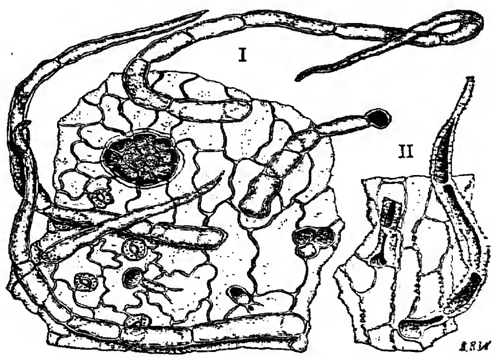
Cayenne



Paprika



Caraway



Sage

Figs. 195–200.

Chemical Composition. The accompanying table shows the minimum, maximum, and average composition of authentic samples of the common tropical spices taken from original packages of importers.[1] Comparable analyses of umbelliferous spices and leaf spices belonging chiefly to the mint family are not available, since in these only the percentages of volatile oil and ash are commonly determined.

Pungent Constituents. On the page following the table of composition are listed the chief flavoring constituents present in the more important spices arranged in the order of the amount or their seasoning value so far as the evidence at hand permits. Unless otherwise stated, the figures are in terms of percentages of the volatile oil obtained by the commercial distillation of the spice. Minor constituents as a rule are omitted. Since quantitative determinations are often difficult and the results approximate or uncertain, it is probable that constituents not yet discovered or classed as minor in the literature may be present in considerable amount. Again a constituent known to be present in a spice grown in a certain region may not be found in the same spice grown in another region. This may be explained as due to a change to a closely related substance during certain stages of growth or because of abnormal climatic conditions.

Terpenes and Related Compounds. Most of the flavoring constituents of the fruit, seed, and leaf spices, present in the volatile (essential) oil obtained by distillation or extraction, are either terpenes or their homologs. For the purpose of bringing out such relationship, representative structural formulas with appropriate legends are given on the page facing the list of spices and their constituents.

General Analytical Methods

The methods given in this section are applicable to all the spices, even though they differ radically as to the nature of the flavoring constituents and the inert matter. For example, cloves contain about 20% of volatile oil and no true starch, whereas ginger contains little volatile oil but about 60% of starch. Such radical differences in composition are strikingly brought out by applying the same methods of analysis.

These same methods also serve to detect many adulterants formerly much used. A starchy adulterant not only reduces the volatile matter, but adds starch, a constituent not present in cloves, mustard, and cayenne. Again a woody material, such as ground cocoanut shells containing no starch, diminishes the starch content below the minimum in pepper and other starchy spices, and increases the fiber in all spices. Although the addition of such foreign material is now rare, the methods are still of importance since they serve to detect the presence of natural impurities, such as pepper shells in black pepper, damaged kernels in nutmegs, and woody stems in various spices, present in undue amount through accident or fraud.

Sample

Grind samples for all determinations, other than of starch, so as to pass a sieve with round holes 1 mm. in diameter. For starch determination by the diastase method in black and white pepper, ginger, allspice, and cloves, grind a portion of the sample to an impalpable powder or at least so as to pass a sieve with round holes 0.5 mm. in diameter.

Water

Richardson Oven Drying Gravimetric Method.[2] Dry 2 g. of the sample in a gas-heated or electrically heated bath at 110° to constant weight.

Subtract from the total loss the weight of volatile oil as determined below. The difference represents moisture.

COMPOSITION OF SPICES

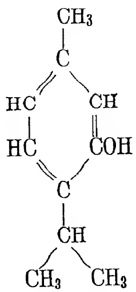
	Water	Protein ($N \times 6.25$)	Oil		Alcohol Extract	Starch		Fiber	Ash		
			Volatile	Fixed		Crude (direct hydrolysis)	Pure (diastase)		Total	Water-soluble	Acid insoluble
	%	%	%	%	%	%	%	%	%	%	%
Allspice											
Min.	9.45	5.19	3.38	4.35	7.39	16.56	1.82	20.46	4.15	2.29	0.00
Max.	10.14	6.37	5.21	7.72	14.27	20.65	3.76	23.98	4.76	2.69	0.06
Aver.	**9.78**	**5.75**	**4.05**	**5.84**	**11.79**	**18.03**	**3.04**	**22.39**	**4.47**	**2.47**	**0.03**
Cassia											
Min.	6.53	3.31	0.93	1.32	4.57	16.65	....	17.03	3.01	0.71	0.02
Max.	11.91	5.44	5.15	4.13	16.74	32.04	....	28.80	6.20	2.52	2.42
Aver.	**9.24**	**4.34**	**2.61**	**2.12**	**8.29**	**23.32**	....	**22.96**	**4.73**	**1.68**	**0.56**
Cassia Buds											
Aver.	**7.93**	**7.53**	**3.88**	**5.96**	**10.88**	**10.71**	....	**13.35**	**4.64**	**2.88**	**0.27**
Cayenne											
Min.	3.67	13.31	0.73	17.17	21.52	7.15	0.80	20.35	5.08	3.30	0.05
Max.	7.08	14.63	2.57	21.81	27.61	9.31	1.46	24.91	5.96	4.93	0.23
Aver.	**5.73**	**13.67**	**1.35**	**20.15**	**24.35**	**8.47**	**1.01**	**22.35**	**5.43**	**3.98**	**0.15**
Cinnamon											
Min.	7.79	3.25	0.72	1.35	9.97	16.65	....	34.38	4.16	1.40	0.02
Max.	10.48	4.06	1.62	1.68	13.60	22.00	....	38.48	5.99	2.71	0.58
Aver.	**8.63**	**3.70**	**1.39**	**1.44**	**12.21**	**19.30**	....	**36.20**	**4.82**	**1.87**	**0.13**
Cloves											
Min.	7.03	5.88	17.82	6.24	13.99	8.19	2.08	7.06	5.28	3.25	0.00
Max.	8.26	7.06	20.53	6.67	15.58	9.63	3.15	9.02	6.22	3.75	0.13
Aver.	**7.81**	**6.18**	**19.18**	**6.49**	**14.87**	**8.99**	**2.74**	**8.10**	**5.92**	**3.58**	**0.06**
Clove Stems											
Aver.	**8.74**	**5.88**	**5.00**	**3.83**	**6.79**	**14.13**	**2.17**	**18.71**	**7.99**	**4.26**	**0.60**
Ginger											
Min.	8.71	4.81	0.96	2.82	3.63	53.43	49.05	2.37	3.61 *	1.73	0.02
Max.	11.72	9.75	3.09	5.42	6.58	62.42	60.31	5.50	9.35 *	4.09	2.29
Aver.	**10.44**	**7.74**	**1.97**	**4.10**	**5.18**	**57.45**	**54.53**	**3.91**	**5.27** *	**2.71**	**0.44**
Mace											
Min.	9.78	6.25	6.27	21.63	22.07	26.77	23.12	2.94	1.81	1.06	0.00
Max.	12.04	7.00	8.65	23.72	24.76	34.42	30.43	3.85	2.54	1.33	0.21
Aver.	**11.05**	**6.47**	**7.58**	**22.48**	**23.11**	**31.73**	**27.87**	**3.20**	**2.01**	**1.13**	**0.07**
Nutmeg											
Min.	5.79	6.56	2.56	28.73	10.42	17.19	14.62	2.38	2.13	0.82	0.00
Max.	10.83	7.00	6.94	36.94	17.38	25.60	24.20	3.72	3.26	1.46	0.01
Pepper, black											
Min.	10.63	10.50 †	0.65	6.86 ‡	8.47	28.15	22.05	10.75	3.09	1.75	0.00
Max.	12.95	13.81 †	1.60	10.37 ‡	11.86	43.47	39.66	18.25	6.52	3.20	1.19
Aver.	**11.96**	**12.05** †	**1.14**	**8.42** ‡	**9.62**	**38.63**	**34.15**	**13.06**	**4.76**	**2.54**	**0.47**
Pepper, white											
Min.	12.72	10.44 †	0.49	6.26 ‡	7.19	56.43	53.11	0.54	1.03	0.28	0.00
Max.	14.47	11.19 †	0.95	7.94 ‡	8.55	64.92	63.60	4.25	2.96	0.80	0.20
Aver.	**13.47**	**10.89** †	**0.73**	**6.91** ‡	**7.66**	**59.17**	**56.47**	**3.14**	**1.77**	**0.47**	**0.10**
Pepper shells	10.57	14.19 †	0.68	3.04 ‡	4.00	11.43	2.30	32.15	11.91	3.20	4.70

* CaO min. 0.20, max. 3.53, aver. **0.80**%. † Total N less N in fixed oil, $\times$ 6.25. ‡ Piperine (N in fixed oil, $\times$ 20.36): black pepper, min. 5.50, max. 8.14, aver. **6.71**; white pepper, min. 5.29, max. 6.92, aver. **6.11**; pepper shells 1.83%.

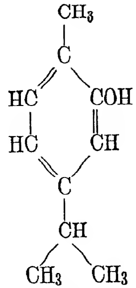
PUNGENT AND ODOROUS CONSTITUENTS OF SPICES

(Percentages are of the volatile oil unless otherwise stated.)

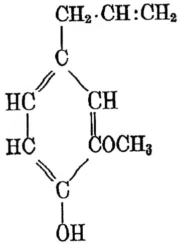
Allspice. *Eugenol, caryophyllene, methyl eugenol, cineol, l-α-phellandrene.*
Anise. *Anethole* 80–90%, *methyl chavicol, aniseketone.*
Bay Leaf. *Cineol* 50%, *geraniol, l-α-terpineol.*
Birch. *Methyl salicylate* 99.8%.
Black and White Pepper. Fixed oil: *piperine, chavicine.* Volatile oil: *piperidine* (?), *β-methylpyrroline.*
Calamus. *Calameone, asarone.*
Caraway. *d-Carvone* 50–60%, *d-limonene* nearly 50%.
Cardamom. *Terpinyl acetate, d-α-terpineol, cineol.*
Cassia. *Cinnamal* 70–90%.
Celery Seed. *d-Limonene, sedanolide* and *sedanonic acid anhydride* (odorous).
Cinnamon. *Cinnamal* (cinnamic aldehyde) 63–84%, *eugenol* 4–12%.
Citronella. *Geraniol* 26–46%, *citronellal* (odorous) 6–54%.
Citrus Oils. See Lemon and Orange Oils.
Clary. *l-Linaloöl, linalyl acetate, sclareol.*
Cloves. Volatile oil: *eugenol* 80–95%. Fixed oil: *caryophyllin, eugenin.*
Coriander. *Coriandrol* (*d*-linaloöl) 60–70%.
Cubebs. Fixed oil: *cubebin.*
Cumin. *Cuminal* (characteristic) 20%, *cymene, hydrocuminene.*
Dill. *Carvone* 40–60%, *d-limonene, phellandrene.*
Fennel. *Anethole* 50%, *fenchone* 20%.
Ginger. Fixed oil: *zingerone* (pungent). Volatile oil: *zingiberene* (sesquiterpene), *d-camphene, β-phellandrene, zingiberol* (odorous).
Japanese Peppermint. *Menthol* 70–92 (free 58–85), *menthone* 11–14%.
Juniper Berry. *α-Pinene.*
Lemon Grass. *Citral* 70–90%.
Marjoram. *Terpenes* (chiefly terpinene) 40%, *d-α-terpineol.*
Mustard. Which see.
Nutmeg and Mace. *d-Pinene* and *d-camphene* 80, *dipentene* 8, *myristicin* 4%.
Orris. *Irone* (odorous), *myristic acid* and *methyl ester* 80–90%.
Paprika and Cayenne. *Capsaicin* 0.03–0.55% of spice.
Parsley Seed. *Apiole* (chief constituent).
Peppermint. *Menthol* 34–86 (free 24–61), *menthone* 8–42%.
Rose. *Geraniol, citronellol, stearoptene.*
Rosemary. *Borneol, α-pinene, camphene, cineol, camphor, bornyl acetate.*
Sage. *Borneol* (chief constituent), *thujone* (chief odorous constituent).
Sassafras. *Safrole* 80, *phellandrene* 10, *d-camphor* 6.8%.
Spearmint. *Ketones* largely *l-carvone* 43–80%, *dihydrocarveol* 18% as acetate.
Star Anise. *Anethole, methyl chavicol, aniseketone.*
Summer Savory. *Carvacrol* (chief odorous principle) 30%.
Sweet Basil. *Methyl chavicol, linaloöl, cineol, ocimene.*
Tarragon. *Methyl chavicol* (estragol).
Thyme. *Phenols* (*thymol* and *carvacrol*) 20–70%.
Tonka, Vanilla. Which see.
Wintergreen. *Methyl salicylate, secondary alcohols, aldehydes,* and *esters.*



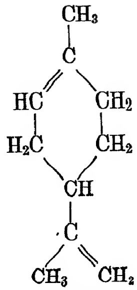
Thymol
(phenol)



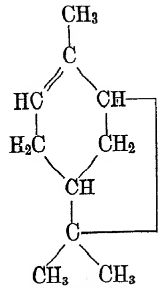
Carvacrol
(phenol)



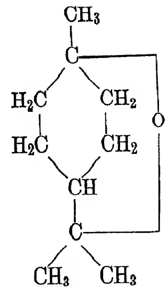
Eugenol
(phenol)



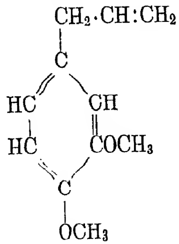
Limonene
(terpene)



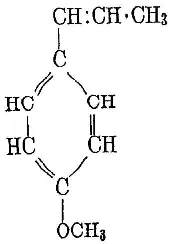
Pinene
(terpene)



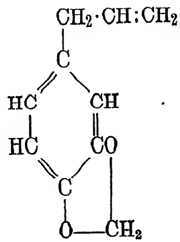
Cineol
(terpene oxide)



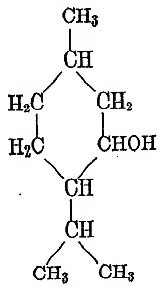
Methyl eugenol
(phenyl ester)



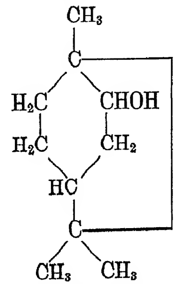
Anethole
(phenyl ester)



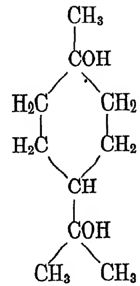
Safrole
(phenyl ester)



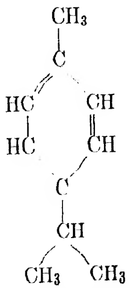
Menthol
(secondary terpene-alcohol)



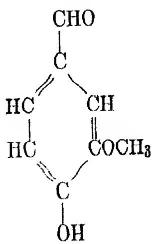
Borneol
(secondary terpene-alcohol)



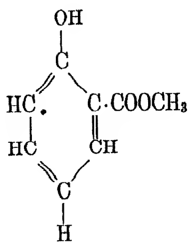
Terpinol
(dihydric tertiary
terpene-alcohol)



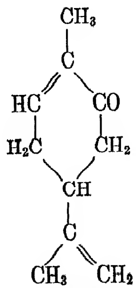
Cymene
(aromatic hydrocarbon)



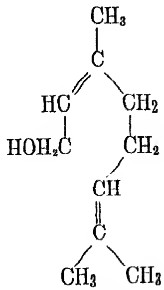
Vanillin
(aromatic aldehyde)



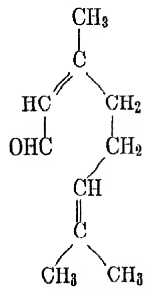
Methyl salicylate
(aromatic ester)



Carvone
(terpene-ketone)



Geraniol
(open-chain primary alcohol)



Citral
(open-chain aldehyde)

Phenols and Related Constituents of Volatile Oils

Terpenes and Related Constituents of Volatile Oils

Scholl and Strohecker Distillation Method.[3] The results by this method on pepper, cinnamon, cloves, anise, saffron, and mustard are said to agree closely with those by the Richardson method, but on mace and caraway they are lower.

APPARATUS. The *Mai and Rheinberger Apparatus* [4] is recommended by Scholl and Strohecker, but the Bidwell and Sterling (Fig. 97) or the Brown and Duvel apparatus (Fig. 96) is adequate.

REAGENT. *Xylene-Toluene Mixture:* 95 ml. of xylene and 5 ml. of toluene.

PROCESS. *Distillation.* Place 30 g. of the sample in a suitable distillation flask, add 200 ml. of the *xylene-toluene mixture*, and distil into a graduated cylinder. The boiling point, which at first is 135°, rises slowly to 139°. Discontinue the distillation when 100 ml. of liquid have passed over.

Reading. Remove the water adhering to the sides of the condenser with a wire, rinsing with the *xylene-toluene mixture*, or causing vapors of the latter to pass over after turning off the water from the jacket. Keep the liquid in the cylinder in a bath at 15° for 3 to 4 hours, to allow the xylene-toluene mixture to clear up, and read the volume of the aqueous column.

Add the xylene-toluene mixture in the receiver to that in the flask, rectify, and use for subsequent determinations. Clean the receiver with oxidizing mixture.

NOTES. Van Itallie, Kerbosch, and Olivier [5] endorse the use of *xylene* and *toluene* and state that benzene yields low and paraffin high results.

The Sindall Distillation Method[6] employs 50 g. of spice and 150 ml. of *kerosene*. The results obtained are considerably higher than by the Richardson method.

PROTEIN

Determine nitrogen in all spices, except black and white pepper (which see), by the Kjeldahl-Willfarth or Kjeldahl-Gunning method and calculate protein by the factor 6.25.

VOLATILE AND FIXED OIL

Richardson Ether Extraction Gravimetric Method.[7] Extract 2 g. of the sample, without drying, for 20 hours with *absolute ether* in a Johnson or other continuous extractor. Transfer the ethereal solution to a flat-bottom aluminum dish 5 cm. in diameter and allow the ether to volatilize at room temperature until no more moisture is visible. Dry over freshly boiled sulfuric acid for 18 hours and weigh the total oil (total ether extract).

Heat first for 6 hours in a boiling water oven, then at 110° to constant weight. The loss is volatile (essential) oil, the residue is fixed (non-volatile) oil.

The following average figures [8] show that after 20 hours of extraction in the Johnson extractor practically all the total ether-soluble matter has been removed.

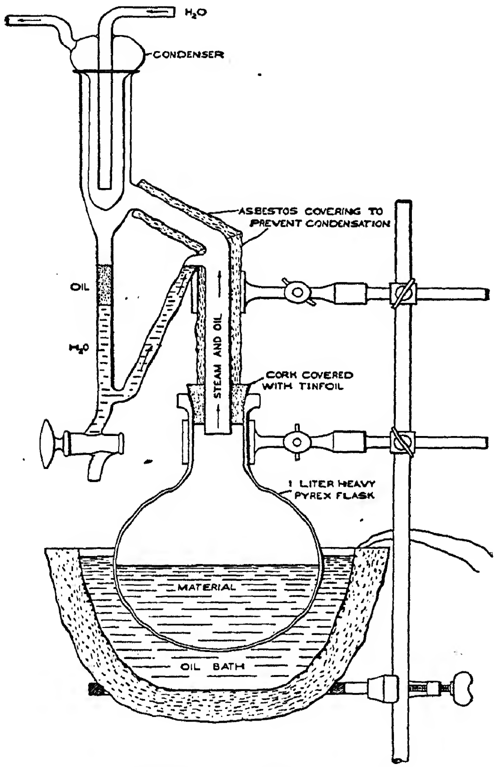
	No. of Samples	First 10 Hours	Second 10 Hours	Third 10 Hours
Allspice		9.28	0.28	0.18
Black pepper		9.40	0.14	0.08
Cassia		5.87	0.27	0.10
Cassia buds		9.37	0.33	0.14
Cayenne		20.59	0.43	0.20
Cloves		25.18	0.25	0.16
Clove stems		8.56	0.16	0.10
Cinnamon		2.63	0.26	0.19
Ginger		4.32	0.18	0.11
Mace		31.53	0.25	0.21
Nutmeg		39.08	0.19	0.11

VOLATILE OIL

Clevenger Distillation Method.[9] Girard and Dupré,[10] among others, describe an analytical process for the determination of volatile oil that differs from the production process only in being on a small scale. The results were at best only approximate and it

was not until Clevenger revived the idea and perfected the apparatus that the distillation procedure could be regarded as strictly quantitative.

APPARATUS. The *Clevenger Apparatus* is for the distillation method what the Johnson



Courtesy of Methods of Analysis, A.O.A.C. 1940, p. 470

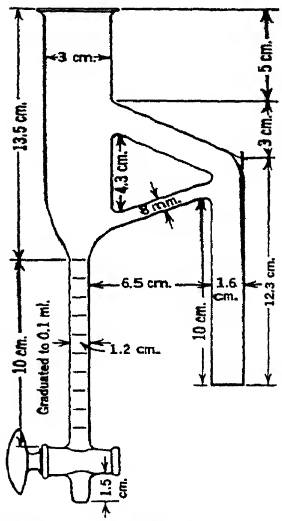
FIG. 201. Clevenger Distillation Assembly for Oils Lighter than Water.

and the Soxhlet apparatus are for the direct extraction method.

Distillation Assembly. The complete apparatus with separator trap for volatile oil lighter than water is shown in Fig. 201. The separator trap for oils heavier than water is shown in Fig. 202.

PROCESS. *Distillation.* Weigh into the 500- to 2000-ml. Pyrex flask an amount of the whole or ground sample, containing preferably 2 ml. or more of volatile oil, add three to six times as many milliliters of water as there are grams of the sample, and mix thoroughly. Place in the oil bath and connect with the separator trap and condenser. Add to the bath a suitable amount of *oil* (*such as hydrogenated cottonseed oil*), apply heat, and boil gently for 4 to 8 hours, or until the volatile oil has been driven off, avoiding the escape of vapors about the condenser.

If the spice (e.g., nutmeg) contains volatile oils lighter and fixed oils heavier than water,



Courtesy of Methods of Analysis, A.O.A.C., 1940, p. 471

FIG. 202. Clevenger Separatory Trap for Oils Heavier than Water.

stop the distillation when the fraction obtained during 1 hour is heavier than water.

Should the separation of the volatile oil be unsatisfactory, draw off the contents of the trap into a small separatory funnel. After the layers separate, return the water to the trap and transfer the volatile oil to the grad-

uated cylinder, repeating the procedure if necessary.

With volatile oils heavier than water, after transferring the oil to the graduated cylinder, run the water together with any remaining oil into a small separatory funnel. Wash the oil trap with 10 ml. of *ether* and transfer the washings to the funnel. Shake, draw off the ether, evaporate the ether, and drain the residue into the cylinder.

Reading. Read the volume of the volatile oil directly in the graduated cylinder and report as milliliters of oil per 100 g. of spice.

Employ the volatile oil after standing until clear, or dried with a minimum amount of *anhydrous sodium sulfate,* for the determination of values.

NOTE. Following Clevenger, other authors including Cocking and Middleton,[11] Markwell,[12] Kofler and von Herrensschwand,[13] and Wasicky, Graf, and Bayer [14] have published papers describing distillation apparatus. In the last two mentioned, saturated sodium chloride is given as the distillation medium.

Brown Combustion Gravimetric Method.[15] This method has found favor in England.

APPARATUS. The following in series: (1) a *Soda-Lime Tube* or other device for removing carbon dioxide from the air; (2) a *U-Tube* of 5- to 10-mm. bore with a thick-walled delivery tube of only 2-mm. bore, heated in an air bath consisting of 2 metal cans, one inside the other; (3) a *Combustion Tube,* containing copper oxide, heated by a furnace into which projects for 10 cm. the delivery tube of the U-tube through a perforated stopper; (4) a *Calcium Chloride U-Tube* containing sulfuric acid and pumice; (5) a *Potash Bulb* such as is used in ordinary combustion; and (6) an *Aspirator.*

PROCESS. Introduce into the U-tube 1 to 5 g. of the material, connect the apparatus, and heat at 100° until the substance is dry, aspirating continually; then slowly raise the heat to the required temperature, which differs for the different spices, and maintain at that temperature for 30 minutes or as long as experience shows necessary.

The weight of water absorbed by the sulfuric acid is not determined, as the originator of the method found that it did not represent accurately the amount of water in the spice.

CALCULATION. The weight of carbon dioxide absorbed by the potash bulb multiplied by a factor, which differs with the spice, gives the percentage of volatile oil.

The following are the temperatures and factors:

	Temperature	Factor
Caraway	130–140	81
Cinnamon	150–160	80
Coriander	140	75
Cardamom	140	73
Anise *	150	77
Black pepper	...	85
Allspice	...	73
Bay leaf	150	76
Juniper	150	83.5

* Deduct 0.1% for each 30 minutes' heating.

The method is not satisfactory for ginger and the factor for cloves is missing.

PHYSICAL AND CHEMICAL VALUES OF VOLATILE OIL

The volatile oil separated from the spice by the distillation method or from an extract by a centrifugal method may be used for the determination of the *refractive index* in the Abbé instrument, the *specific gravity* in a micro Sprengel pycnometer, and the *optical rotation* in a polarizing tube of small diameter. In reporting the results, the direct reading of optical rotation, α_D, is preferable to specific rotation, $[\alpha]_D$, which requires needless calculation, the temperature of observation being added over the D. Widespread improper use of the brackets in the literature has resulted in much confusion.

The *iodine number* is of little significance in the examination of volatile oils, but the

acid number and the *ester number*, which is the saponification number determined on the neutralized portion after titration for the acid number, and the *ester number after acetylation* are important values. The latter term may well give place to *acetyl number* to correspond with the usage of fatty oil chemists. It is expressed in terms of milligrams of potassium hydroxide per gram of oil, as is also the acid number, and represents the total alcohols combined as esters displaced by the alkali. In the conventional method, boiling with acetic anhydride in the presence of anhydrous sodium acetate converts the alcohols into acetic esters (acetates).

If the ester (or esters) of an alcohol of the $C_{10}H_{18}O$ (or $C_{10}H_{20}O$) group is known to predominate, the percentage of the acetate (E), that is, the acetic ester of that alcohol, and of the combined (as ester) alcohol (C) may be calculated from the ester number (K). Given the acetyl number (A), the total alcohol (T) and the free alcohol (F) may also be calculated in terms of the predominating alcohol. The formulas for terpineol, geraniol, linaloöl, borneol, $C_{10}H_{18}O$ (or menthol, citronellol, $C_{10}H_{20}O$) follow:

$$E = 0.35(\text{or } 0.3536)K$$

$$C = 0.275\,(\text{or } 0.279)K$$

$$T = \frac{7.7(\text{or } 7.8)A}{28 - 0.021A}$$

$$F = T - C$$

$$K = 2.857\,(\text{or } 2.828)E$$

$$= 3.636\,(\text{or } 3.584)C$$

$$A = \frac{28T}{7.7\ (\text{or } 7.8) + 0.021T}$$

Much calculation may be avoided by consulting Gildermeister and Hoffmann tables for alcohols of different molecular weights showing the following equivalents: (1) ester number, (2) percentage of acetate, (3) percentage of alcohol in the acetylated oil, and (4) percentage of alcohol in the original oil corresponding to (3).

French oil chemists substitute *ester number after formylation*, or *formyl number*, for ester number after acetylation (acetyl number). In the foregoing calculation formulas, 0.014 is substituted for 0.021.

Alcohol Extract

Winton, Ogden, and Mitchell Cold Digestion Method. Weigh 2 g. of the sample into a 100-ml. volumetric flask and make up to the mark with *ethanol*. Stopper, shake, and allow to stand 24 hours, shaking every 30 minutes during the first 8 hours. Filter through a dry paper, pipet 50 ml. into a flat-bottom aluminum dish, 5 cm. in diameter, evaporate to dryness, and dry to constant weight at 110°.

The results of this official method are empirical and of value chiefly in detecting previous extraction of ginger for the preparation of ginger extract.

Sugars

Determine reducing sugars (Part I, C6*a*) in a dealcoholized aliquot of the washings obtained in the starch determination, or preferably of a new solution prepared by weighing 10 g. of the sample into a 250-ml. flask, adding water to the mark, shaking at intervals, and filtering through a dry paper.

Crude Starch

(Copper Reducing Matter by Direct Hydrolysis)

Sachsse Direct Hydrolysis Copper Reduction Gravimetric Method. See Part I, C6*a*. The method is official.

In pepper, ginger, nutmeg, and mace containing much starch (in mace amylodextrin starch) but little or a moderate amount of fiber, the results by direct hydrolysis are not

considerably higher than by the diastase method and may well be designated *crude starch.* On the other hand, in cayenne pepper containing no true starch but much fiber, in cloves also containing no true starch but much tannin matter, and in allspice containing only a small amount of starch but much tannin matter and fiber, the results by direct hydrolysis are several times as high as by the diastase method and should be designated *copper reducing matter by direct hydrolysis calculated as starch.*

Since Batavia cassia and probably some other varieties form a gelatinous mass that clogs completely the filter on washing with water or 10% ethanol, it is recommended to omit washing with sugar solvents in the preliminary treatment of all cassias, cassia buds, and cinnamons. If it is desired to correct for sugars, determine this in a separate portion as directed above.

Pure Starch

Märcker Diastase Digestion Copper Reduction Gravimetric Method. Reduce a portion of the sample to an impalpable powder or at least so as to pass a sieve with round holes 0.5 mm. in diameter and follow the directions given in Part I, C6*a*. The method is official.

The small percentages of starch in cayenne and cloves by this method given in the table above do not represent true starch but small amounts of other reducing substances such as sugars and tannins. Neither of these spices gives reactions for starch.

Fiber

See Part I, C2*e*.

Ash

Total Ash, Water-Soluble Ash, Acid-Insoluble Ash, Alkalinity of Ash. See Part I, C2*f*. The methods are official.

Special Analytical Methods

To this class belong methods of determination of constituents peculiar to individual spices, such as piperine in pepper and volatile mustard oil in mustard flour. Other methods, not here given, but found in works on essential oils,[16] are for individual constituents of the essential oils, such as eugenol in cloves and menthol in peppermint.

a. Allspice and Cloves

The soluble members of this group, although of no appreciable food value, contribute to the flavor of cloves and allspice, even though the astringency is masked by the pronounced pungency of the terpenes. As is true of tannin in the apple, the astringent flavor, of itself disagreeable, in combination doubtless adds a pleasing touch.

Tannins are characterized by their strong affinity for oxygen, as measured by the oxygen absorbed from potassium permanganate solution, also by their copper reducing power. This latter property explains why the crude starch (reducing matter by direct hydrolysis) of cloves is several times that of the pure starch, although the fiber is relatively low (about 6%), whereas the crude starch of black pepper, with a higher fiber content but little or no tannin, approaches that of the pure starch.

Löwenthal-Richardson Permanganate Volumetric Method.[17] The method, originally designed for tannin material, has been simplified by Richardson for the analysis of cloves and allspice. The use of hide powder as a reagent is omitted.

Reagents. *Standard Potassium Permanganate Solution.* Dissolve 1.333 g. of $KMnO_4$ in water and dilute to 1 liter. Standardize against 10 ml. of standard *0.1 N* oxalic acid solution which is equivalent to 0.06235 g. of

quercitannic acid or 0.008 g. of oxygen absorbed.

Indigo Solution. Dissolve 6 g. of sodium sulfindigotate in 500 ml. of water with the aid of heat, cool, add 50 ml. of H_2SO_4, dilute to 1 liter, and filter.

PROCESS. *Extraction and Solution.* Weigh 2 g. of the sample into the inner tube of a Johnson extractor or a cylinder formed by folding a round piece of filter paper over the flat bottom of a shell vial. Extract for 20 hours with *absolute ether*, allow the ether to evaporate from the extracted residue, and transfer the latter to a 500-ml. volumetric flask. Add 300 ml. of water, boil for 2 hours, cool, make up to the mark, and filter through a dry paper. Pipet 25 ml. of the filtrate into a flask of about 1200 ml. capacity.

Titration. To the 25-ml. aliquot, add 20 ml. of *indigo solution* and 750 ml. of water, then run in *standard potassium permanganate solution*, 1 to 2 drops per second with shaking, until the color is bright golden yellow.

Blank. Titrate 20 ml. of the indigo solution diluted with 750 ml. of water in like manner.

CALCULATION. Multiply the difference between the two titrations by the weight of quercitannic acid or oxygen absorbed equivalent to 1 ml. of potassium permanganate solution, as standardized against 0.1 N oxalic acid.

b. Cayenne and Paprika

IODINE NUMBER OF EXTRACTED OIL

Doolittle and Ogden [18] suggested extraction with ether and determination of the iodine number of the extract as a means of detecting foreign oil in paprika added to intensify the color. Extraction in a continuous apparatus having failed to give satisfactory results, a conventional method was suggested by Winton [19] and elaborated by Seeker [20] essentially as follows.

Seeker Ether Digestion Iodometric Method. Remove the moisture from 5 g. of the sample by allowing to stand over sulfuric acid for 12 hours. Fill a 250-ml. volumetric flask to the mark with *ethanol-free ether* and brush the dried sample into the liquid. Shake and repeat the shaking at intervals of 20 minutes for 1 hour. Pipet 100 ml. of the clear supernatant liquid into a tared 250-ml. glass-stoppered flask, distil the ether, dry in a boiling water oven to constant weight, and determine the iodine number by the Hanus Method. See Part II, B2*b*.

In the present official method of the A.O.A.C.,[21] the error of weighing so large a flask and the difficulty of complete drying of the oil therein is avoided by measuring one of two aliquots of 10 ml. of a chloroform extract, representing 1 g. of the material, into the flask and the other into a dish for evaporation and drying to determine the weight of oil for use in the calculation of iodine number.

CAPSAICIN

von Fodor Vanadium Colorimetric Method.[22] As proposed by von Fodor for quantitative work, a *1% solution of vanadyl trioxychloride* ($VOCl_3$) in *carbon tetrachloride* is added to an ether extract of the sample, prepared by shaking with *ether* and centrifuging, and the color is compared with that formed with a *standard solution* prepared in the same manner. The color formed varies from blue, through green, to brown-green for concentrations of capsaicin ranging respectively from 0.08 to 0.01%.

Vanadyl trioxychloride capsaicin has the formula $C_{18}H_{26}NO_3 \cdot VOCl_2$.

In making the qualitative test, von Fodor prefers an *acetone* extract of the capsaicin and *ammonium metavanadate* (NH_4VO_3) as the reagent.

NOTE. Tice [23] further developed the method.

Prokhorova and Prozorovskaya Modification.[24] The only material difference between the modification and the original quantitative method is that the sample is extracted with freshly distilled acetone and the color comparison is made in a colorimeter.

APPARATUS. *Colorimeter.*

REAGENT. *von Fodor Vanadium Reagent:* 1% vanadyl trioxychloride in CCl_4.

PROCESS. *Extraction.* Shake 2 g. of the ground sample with exactly 100 ml. of freshly distilled *acetone* for 24 hours and allow to settle 1 hour.

Color Formation. To 5 ml. of the clear solution, add dropwise *von Fodor vanadium reagent* until no further color change is evident.

Color Comparison. Make the comparison in a colorimeter with a *standard 0.016% capsaicin solution* to 5 ml. of which have just been added 20 drops of the *reagent* with mixing.

EXAMPLES. The maximum content was found in the inner walls of the fruit and practically none in other parts of the fruit and in the seeds.

c. Cinnamon and Cassia

CINNAMIC ALDEHYDE

von Fellenberg Sulfuric Acid Colorimetric Method.[25] APPARATUS. *Colorimeter.*

REAGENTS. *Isobutanol,* 5%, in ethanol. It should give no color with a double volume of H_2SO_4.

Standard Cinnamic Aldehyde Solution, 2%. Dissolve in 1 liter of 38% ethanol a quantity of cinnamic aldehyde-sodium bisulfite, equivalent to 20 divided by its cinnamic aldehyde content, and titrate against 0.02 N bromine or 0.02 N iodine solution. Since 1 liter of 1.0 N bromine or iodine solution is equivalent to half the molecular weight of cinnamic aldehyde, it follows that

$$^{132.1} \times 1000 \times (B \text{ or } I)$$

in which C is the cinnamic aldehyde content of the aldehyde-bisulfite, B is the milliliters of 0.02 N bromine solution, and I the milliliters of 0.02 N iodine solution.

PROCESS. *Distillation.* Distil 1 g. of the finely ground sample in a 400-ml. flask with 40 ml. of *ethanol,* heat at first gently for 10 minutes, and then boil so as to distil all but 5 to 10 ml. of the ethanol, avoiding burning the residue. To the distillate add 100 ml. of recently boiled water and distil until the distillate measures 100 ml. in a volumetric flask.

Color Formation. Mix the distillate, place 5 ml. in a 100-ml. graduated flask, then add 2 ml. of *5% isobutanol in ethanol* and 3 ml. of *38% ethanol,* run in 20 ml. of *sulfuric acid,* and shake.

Color Comparison. After allowing to stand 30 minutes alongside of a solution prepared by treating in like manner 5 ml. of a *standard 2% cinnamic aldehyde solution,* compare in a colorimeter, using *2 + 1 sulfuric acid* for dilution if necessary.

CALCULATION. Multiply the color strength of the solution as found by 2 and obtain the percentage of cinnamic aldehyde from the following table:

Color Strength	Cinnamic Aldehyde %	Color Strength	Cinnamic Aldehyde %
0.2	0.24	1.8	1.85
0.4	0.45	2.0	2.00
0.6	0.65	2.2	2.18
0.8	0.86	2.4	2.35
1.0	1.07	2.6	2.52
1.2	1.28	2.8	2.70
1.4	1.48	3.0	2.90
1.6	1.67	3.2	3.09

d. Ginger

COLD WATER EXTRACT

Winton, Ogden, and Mitchell Method.[26] Determination of cold water extract detects exhaustion of ginger with water for the manufacture of ginger ale; determination of alcohol extract detects exhaustion for the preparation of ginger extract.

PROCESS. Weigh 4 g. of the sample into a 200-ml. volumetric flask, add water to the mark, stopper, and shake every 30 minutes for 8 hours. Allow to stand 16 hours without shaking and filter. Transfer an aliquot of 50 ml. to a tared flat-bottom aluminum dish, 5 cm. in diameter, evaporate to dryness on a water bath, and dry in a boiling water oven to constant weight, 2 hours usually being sufficient.

e. Black and Brown Mustard

VOLATILE MUSTARD OIL

Formation. The seeds of different species of mustard yield differing percentages of a pungent volatile oil consisting essentially of allyl isothiocyanate. Winton and Bornmann[27] (Chicago) report on mustard seeds as follows: black (*Brassica nigra*) 1.57, brown (*B. Besseriana*) 0.98, white (*Sinapis alba*) 0.05, and wild (*Brassica arvensis*) 0.07%.

The volatile oil (*allyl isothiocyanate*) does not exist ready formed in the seed, but is produced by the action of the enzyme *myrosinase* (myrosin) on *sinigrin* (potassium myronate) by the following reaction:

Potassium myronate

$$C_6H_{12}O_6 + C_3H_5CNS + KHSO_4$$

Allyl isothiocyanate

Roeser Silver Nitrate Volumetric Method.[28] PROCESS. *Digestion.* Weigh 5 g. of the ground sample into a 200-ml. distilling flask, add 60 ml. of water and 15 ml. of *60% ethanol*, then digest for 2 hours at room temperature.

Distillation. Connect with a distilling assembly and distil into a 100-ml. volumetric flask containing 10 ml. of *10% ammonium hydroxide*, into which the extension tube dips, until about 50 ml. have been collected.

Precipitation of Silver Sulfide. To the distillate, add 10 ml. of *0.1 N silver nitrate solution* and shake. After allowing to stand for 24 hours, make up to the mark and filter through a dry paper.

Titration. Pipet 50 ml. of the filtrate into a beaker, add 5 ml. of *standard 0.1 N potassium cyanide solution*, and titrate back with *standard 0.1 N silver nitrate solution*, using *slightly ammoniacal 5% potassium iodide solution* as indicator.

CALCULATION. Use the same calculation factor as for the Godamer-Kuntze method below, as recommended by Bouton.[29]

Roeser divides by 0.93×5 instead of 5, thus calculating the percentage of volatile mustard oil containing 93% of allyl isothiocyanate.

Godamer-Kuntze Silver Nitrate Volumetric Method.[30] REAGENT. *Standard Ammonium Thiocyanate Solution*, 0.1 *N*. Adjust to exactly tenth normal, titrating against 0.1 *N* $AgNO_3$ solution and using $FeNH_4(SO_4)_2 \cdot 12H_2O$ as indicator.

PROCESS. *Digestion.* Weigh 5 g. of the ground sample into a 200-ml. stoppered flask and digest for 2 hours at 37° with 100 ml. of water.

Distillation. Add to the mixture 20 ml. of *ethanol* and distil at once into a 100-ml. volumetric flask containing 10 ml. of *10% ammonium hydroxide*, into which an extension of the condenser tube dips, until 60 ml. have been collected. Allyl isothiocyanate forms, with the ammonia, thiosinamine, $C_3H_5 \cdot NH \cdot CS \cdot NH_2$.

Silver Sulfide Precipitation. To the distillate, add 20 ml. of *standard 0.1 N silver nitrate solution* and allow to stand overnight.

Heat the mixture to boiling to flock the precipitate, cool, make up to 100 ml., shake, and filter through a dry paper.

Titration. To 50 ml. of the filtrate, add 5 ml. of *nitric acid* and titrate with *standard 0.1 N ammonium thiocyanate solution,* using 5 ml. of *10% ferric ammonium sulfate solution* as indicator.

CALCULATION. To obtain the percentage of allyl isothiocyanate, subtract the milliliters required for the titration from 20 and multiply by $0.004957 \times 2 \times (100/5)$ or, simplified, by 0.1983.

Gros and Pichon Modification.[31] The digestion is carried out at 40° *in vacuo* for only 15 minutes, followed by distillation in a bath at 65 to 70° for about 40 minutes. The materially shorter time required for the operation is, however, offset by the more complicated equipment. The absorption pipet is described (but not illustrated) and working details are given in *Chemical Abstracts*.[32]

Madis Bromide Volumetric Method.[33] Although devised for commercial mustard oil, the method may be applied to the distillate collected in ammonium hydroxide as in the foregoing methods. The directions which follow are for the oil.

PROCESS. *Treatment with Ammonium Hydroxide.* To 0.12 g. of the oil in a 100-ml. volumetric flask, add 5 ml. of *ethanol* and 5 ml. of *10% ammonium hydroxide.* Place a funnel in the neck of the flask and heat for 10 minutes, then cool, make up to the mark, and remove 10 to 20 ml. (12 to 24 mg.) with a pipet. These amounts are about the same as in the whole distillate by the foregoing methods.

Bromide Treatment. Dilute the aliquot to 40 ml. with water, add 2 g. of *potassium bromide* and 1 ml. of *1% auric bromide solution,* then *hydrochloric acid* to acid reaction, and heat to 45°.

Bromate Titration. Add to the colorless solution *standard 0.1 N potassium bromate solution* until a permanent yellow color appears.

CALCULATION. Use the formula: 1 ml. of 0.1 *N* potassium bromate = 0.001277 g. of allyl isothiocyanate.

f. White Mustard

SINALBIN

White mustard, although practically free from volatile oil, contains a glucoside, sinalbin, which by enzyme hydrolysis splits into sinalbin mustard oil (*p*-hydroxybenzyl isothiocyanate), dextrose, and sinapin hydrogen sulfate thus:

Sinalbin

$C_6H_{12}O_6$ ·

Sinalbin mustard oil

Sinapin hydrogen sulfate

Wild mustard or charlock yields neither allyl isothiocyanate nor sinalbin mustard oil in considerable amount and is regarded as an adulterant if used in mustard flour or prepared mustard.

Bauer and Holle Incubation Gravimetric Method.[34] Parallel digestions are made, one with, the other without, initial destruction of the enzyme myrosinase (myrosin) and the amount of sulfate formed by the enzyme in the latter case is obtained by difference.

REAGENT. *Acid Zinc Acetate Solution.* Dissolve 230 g. of zinc acetate in water, add 50 ml. of acetic acid, and dilute to 1 liter.

PROCESS. *Digestion.* Weigh out two 10-g. charges of the sample, extract each with *ether,* and transfer the extracted residue to 500-ml. volumetric flasks.

To one flask add 350 ml. of boiling water, mix, and heat with frequent agitation for 1 hour in a bath of boiling water. Wash down particles on the sides with water at 60°.

To the other flask add 350 ml. of water at 60°, heat in a bath at 60° for 2 hours. Wash down the sides with water at 60°.

Allow both flasks to stand overnight at room temperature.

Ferrocyanide Treatment. Add to each flask 10 ml. of *15% potassium ferrocyanide solution* and 10 ml. of *acid zinc acetate solution*, dilute to the mark, mix, and filter through a dry pleated paper.

Barium Chloride Precipitation. Pipet 100 ml. of each filtrate into a dry beaker, add exactly 25 ml. of *10% hydrochloric acid* and 25 ml. of *standard 0.1 N barium chloride solution*, stir, and allow the barium sulfate to settle at least 30 minutes with occasional agitation, then add a little *kieselguhr* and filter through a dry paper into a dry flask.

Removal of Barium. Transfer 100 ml. of each filtrate to a beaker (equivalent to 1.33 g. of the sample) and determine the excess of barium chloride with *sulfuric acid* in the usual manner.

CALCULATION. Subtract the weight of barium sulfate in the filtrate corresponding to the portion incubated at 60° from that in the filtrate corresponding to the portion heated to boiling. The difference is the weight of barium sulfate derived from the sinapin hydrogen sulfate formed in accordance with the equation given above. Multiply the difference by the factor 3.532 (= molecular weight of sinalbin ÷ molecular weight of barium sulfate = 824.4/233.42), thus obtaining the corresponding weight of sinalbin.

g. Mustard Flour

Ground mustard seed, after removal of the hulls, is known as mustard flour. Black, brown, and white mustards are considered to be the legitimate raw materials for the preparation of the flour, but charlock, a weed separated from screenings, is deficient in desirable pungent constituents.

VOLATILE MUSTARD OIL

See Black Mustard ab

SINALBIN

See White Mustard above.

TOTAL SULFUR

Methods are given in Part I, C8*a*.

CHARLOCK

Modified Waage Acid Chloral Hydrate Test.[35] REAGENT. *Chloral Hydrate Reagent.* Dissolve 16 g. of $CCl_3 \cdot CHO \cdot H_2O$ in a mixture of 10 ml. of water and 1 ml. of HCl.

PROCESS. Mount 10 mg. of the material on a microscopic slide in a drop of *chloral hydrate reagent*, heat gently, and examine under a lens. In the presence of charlock, the black substance in the palisade cells becomes bright carmine.

NOTE. Winton [36] has shown that the color change is due to the acid, chloral hydrate merely acting as a penetrating agent. He obtained the same color change with glycerol or zinc chloride solution containing 5% of hydrochloric acid or with sirupy phosphoric or citric acid. With freshly dissolved chloral hydrate solution containing no added acid, as prepared by Waage, no color reaction was evident, but on standing for some days the solution became acid and reacted like the reagent prepared as above described.

COLOR

Turmeric is detected by microscopic examination and the boric acid test, other colors (nitro colors, tropeolins, etc.) by dyeing tests. See Part I, C11 and 12.

h. Prepared Mustard

The solid ingredients of prepared mustard are mustard flour, ground mustard, various other spices, salt, and sugar, the liquid con-

stituent is vinegar, hence the Official Methods for the determination of solids, ash, salt, ether extract, protein, acidity, colors, and preservatives are essentially those given in Part I, as well as in Part II, using, however, 5 or 10 g. to compensate for the moist condition.

Sugars

Copper Reduction Gravimetric Method. Remove the oil from 10 g. of the sample by shaking and centrifuging with several portions of 80 ml. each of *naphtha*, following the usual technique, then transfer the residue to a 100-ml. volumetric flask, clarify, dilute to volume, filter, and determine sugars before and after inversion by the Munson and Walker Method, Part I, C6*a*.

Reducing Substances by Direct Hydrolysis

Sachsse Direct Hydrolysis Copper Reduction Method. See Part I, C6*a*.

Prepared mustard made solely from the constituents named above, but not subjected to cooking, contains only traces of ready-formed copper-reducing substances which are derived from the spices. If, however, the product is cooked after adding the sugar and vinegar or if glucose is used for sweetening, the amount is considerable. Starch, gelatinized by heating, passes wholly into dextrose by hydrolysis as carried out by the Sachsse method.

Starch

Sullivan Calcium Chloride Iodine-Iodide Copper Reduction Gravimetric Method. See Part I, C6*a*.

Field Modification.[37] Reagents. *Calcium Chloride Reagent.* Adjust a saturated solution to about 0.025 *N* alkalinity.

Ethanol-Alkali Solution: 70 ml. of ethanol plus 25 ml. of 0.01 *N* NaOH solution.

Iodine-Potassium Iodide Reagent. Dissolve 2 g. of iodine and 6 g. of KI in 100 ml. of water.

Process. *Calcium Chloride Treatment.* Weigh 3 to 4 g. of the sample and pipet 50 ml. of the *calcium chloride reagent* into a 500-ml. Erlenmeyer flask. Wet the inside of a reflux condenser with 50 ml. of water, draining 1 minute, connect the flask, and boil gently for 15 minutes. Cool the flask in a pan of water, then add 50 ml. of water from a pipet through the top of the condenser. Remove the flask, stopper, shake vigorously, transfer to a centrifuge bottle, and whirl for 20 minutes.

Ethanol Precipitation. Pipet 50 ml. of the partly clarified middle layer into a centrifuge bottle containing 150 ml. of *ethanol*, mix, and whirl 5 minutes or until clear. Decant the liquid through an asbestos pad in a Caldwell crucible, using suction, transfer the pad to the centrifuge bottle, and rinse adhering particles into the bottle with warm water. Add water to about 100 ml., break up the precipitate by shaking, then add 2 ml. of the *iodine-potassium iodide reagent* and 30 ml. of *saturated ammonium sulfate solution*, mix well, and centrifuge. Filter with suction through an asbestos pad as before, wash the precipitate and the filter with *ethanol-alkali solution* until practically no more blue color is removed, then with a few milliliters of *ethanol*.

Return the pad to the bottle, rinse the crucible with about 20 ml. of water, add 5 ml. of *hydrochloric acid*, and stir to break up the pad. Heat on a steam bath 30 minutes, stirring occasionally. Filter with suction into a clean flask and rinse the bottle and filter with small portions of warm water. Rinse the filtrate into a Kjeldahl flask, boil down to 60 to 70 ml., then reflux for 30 minutes. Cool, rinse into a 100-ml. volumetric flask, nearly neutralize with *sodium hydroxide solution*, and dilute to volume.

Determine dextrose by the Munson and Walker Method, Part I, C6*a*.

Calculation. Weight of dextrose × 0.9 = starch.

Fiber

Saponification of the fat by alkali boiling is not complete and removal by evaporation and dry extraction is not feasible because the product dries to a hard mass. Winton and Andrew [38] remove the fat from the fiber after the acid and alkali boiling by washing with *ethanol* followed by *ether*. In the present Official Method, one of two procedures is directed: (1) shake vigorously 10 g. of the sample and 50 ml. of *ethanol*, add 40 ml. of *ether*, shake, let stand 5 minutes with occasional shaking, centrifuge, and decant, repeating twice, then allow the ether to evaporate from the centrifuge bottle resting on its side or (2) mix in a beaker with *ethanol* and *ether*, filter, and continue the washing with ether. In either case transfer to an Erlenmeyer flask and proceed as in the usual method, Part I, C2*e*.

i. Black and White Pepper

Kjeldahl-Arnold Copper Sulfate Method. See Part I, C1*c*. This method gives the full percentage of nitrogen in black, white, and long pepper and their products. The results agree with those by the Dumas copper oxide combustion method, whereas those by the Kjeldahl-Willfarth or Kjeldahl-Gunning method are 1 to 2% low.

Piperine

Winton, Ogden, and Mitchell Ether Extract Nitrogen Factor Method.[39] Weigh 10 g. of the sample directly (without drying) into a Johnson or other continuous extractor and extract for 20 hours with *ether* into a tared 250-ml. flask made of glass suitable for Kjeldahl digestion. Distil or evaporate the ether, dry first at 100° and then at 110° to constant weight. Determine nitrogen in the residue by the Kjeldahl-Arnold Method (Part I, C1*c*).

Calculate the crude piperine by the factor 20.36, or express result as parts of nitrogen per 100 parts of non-volatile ether extract.

j. Nutmegs, Ginger, and White Pepper

Calcium Oxide

Certain grades of nutmegs, ginger, and white pepper are limed, partly, it is claimed, to ward off attacks of insects and partly to improve the appearance. The maximum amount of CaO (3.53%), given in the table at the beginning of Part II, J1, was in a sample of limed Jamaica ginger, whereas in unlimed ginger only fractions of 1% were present.

Method. Follow the method for ash analysis (Part I, C8*a*), weighing only the ignited calcium oxide.

k. Vanilla Bean

The nature and constituents of the bean and its substitutes are considered under Vanilla Extract below.

Vanillin

Modified Hess and Prescott Ether-Ammonia Gravimetric Method. Macerate 5 g. of the ground or minced beans with 50 ml. of *60% by volume ethanol* and allow to stand overnight. Drain off the liquid, grind the residue, again macerate with 50 ml. of *60% ethanol*, and allow to stand overnight. Filter and wash by decantation and on the paper. Combine all the extracts and washings, dealcoholize, and determine vanillin as directed under Vanilla Extract below.

l. Tonka Bean

COUMARIN

The nature of the tonka bean and the constitution of coumarin are considered under Vanilla Extract, below.

Modified Hess and Prescott Method. Extract 5 g. of the ground beans, as directed for Vanilla Bean above, and determine the coumarin as directed under Vanilla Extract below, omitting the treatment with ammonium hydroxide for the separation of vanillin.

Meyer Nitroaniline Colorimetric Method.[40] The method is designed for the determination of coumarin in tonka beans, but the principle may be applied to the analysis of tonka extract and coumarin flavors.

APPARATUS. *Photometer.*

REAGENT. *Diazotized Reagent (p-Nitroaniline Solution).* Diazotize a solution of 3.5 g. of *p*-nitroaniline in 500 ml. of water.

PROCESS. *Distillation.* Prepare an extract, using *80% ethanol* or *ether.* Add 100 ml. of water and 0.2 ml. of *sulfuric acid* to 50 ml. of the ethanol extract or 25 ml. of the ether extract and distil in a current of steam. Dilute the extract to 1 liter (ethanol extract) or 500 ml. (ether extract) in a volumetric flask.

Color Formation. Transfer 2 to 3 ml. of the extract to a 50-ml. volumetric flask containing 25 ml. of water, add 5 ml. of *2% sodium carbonate solution,* and heat on the steam bath for 5 to 10 minutes. Cool, add 5 ml. of the *diazotized reagent,* and dilute to the mark.

Color Reading. After 10 minutes, determine the color value photometrically or compare the color with that of standard solutions of coumarin treated in like manner.

EXAMPLES. Tonka beans, maximum about 2.3% of coumarin.

m. Plants

COUMARIN

Duncan and Dustman Permanganate Volumetric Method.[41] Finding that the methods designed for extracts are not suited for fodder plants, such as species of *Melilotus,* Duncan and Dustman (West Virginia Agricultural Experiment Station) propose the following.

PROCESS. *Distillation.* Steam distil 5 g. of the sample, ground to pass a 1-mm. sieve, with 80 ml. of water, collecting the distillate, after passing through a 63-cm. Allihn condenser, in a 1-liter suction flask, connected with a manometer. Pass dry steam through the system for 1 or 2 minutes until the mixture reaches vigorous boiling, then reduce the pressure to 140 mm. and continue the distillation to dryness. Rinse the connection bulb, inlet tube, and sides of the distilling flask with 80 ml. of water and redistil as before, repeating the operation until six distillates have been collected.

Lead Precipitation. Make the combined distillates up to 1 liter in a volumetric flask, remove a 50-ml. aliquot, treat with 4 ml. of *0.25 M lead acetate solution,* mix, cover, and allow to stand overnight. Remove the excess of lead with *0.17 M disodium phosphate solution* added in an equivalent amount, as determined by a previous titration, plus 0.5 ml. in addition, and allow to settle for 30 minutes.

Zinc Sulfate Treatment. Add 25 ml. of *20% zinc sulfate solution,* which volume is usually the same as that of the permanganate solution added in the next stage. After allowing to stand at least 4 hours, centrifuge and decant the supernatant liquid from the combined precipitates into a 500-ml. Erlenmeyer flask, then wash twice with 25 ml. of water.

Titration by the Obermayer Method.[42] Combine solution and washings, add an excess of *0.1 N potassium permanganate solution* (usually 25 ml.), dilute to 150 ml., and heat on an asbestos board for 10 minutes. Filter the hot solution through a Gooch crucible, wash, then decompose the excess of permanganate with a known amount of *standard 0.1 N oxalic acid solution,* added in excess. Mix

immediately with 25 ml. of *2 N sulfuric acid* and titrate back with *standard 0.1 N potassium permanganate solution*, maintaining the temperature at 70 to 80°.

CALCULATION. The reaction involved in the titration is as follows:

$$3C_9H_6O_2 + 38KMnO_4 \longrightarrow 38MnO_2 + 19K_2O + 27CO_2 + 9H_2O$$

Since the titration accounts for only 93% when pure coumarin is used, a corresponding correction is necessary.

COUMARIN AND MELILOTIC ACID

Roberts and Link Diazonium Colorimetric Method.[43] This method was devised by Roberts and Link (University of Wisconsin) in connection with breeding experiments designed to reduce to a minimum the unpalatable principles of fodder plants.

APPARATUS. *Incubation Tubes*, of Pyrex glass 10 x 75 mm., fitted with a cork carrying a pestle made from a glass rod.

Side-Arm Filtration Tubes, 18 x 150 mm., graduated at 10 ml. and fitted with a cork carrying a small long-stem Büchner funnel.

Color Comparison Tubes, in a comparator.

REAGENTS. *Diazonium Reagent.* (1) Dissolve 3.5 g. of *p*-nitroaniline in 45 ml. of 37% HCl, dilute to 500 ml. with water, and filter. This solution keeps indefinitely if stoppered. (2) Dissolve 5 g. of $NaNO_2$ in 100 ml. of water. Store in the dark and renew often. Chill a 100-ml. volumetric flask, also both solutions, in chipped ice. Add 3 ml. each of solutions 1 and 2 to the flask, chill for 5 minutes, then add 12 ml. of solution 2, mix, chill again for 5 minutes, and fill to the mark with ice-cold water. Mix and chill for 15 minutes before using. If kept well chilled, the solution keeps at least 24 hours.

Color Standard. To 12 color comparison tubes, add aqueous coumarin solution, containing 4 γ of coumarin per milliliter, in 2-γ increments from 0 to 22 γ, then add to each 1 ml. of 1% sodium carbonate solution and water to a volume of about 6 ml. Mix by inverting, heat in a bath at 85° for 5 minutes, cool to room temperature, and add 1 ml. of diazonium reagent dropwise with mixing. Dilute to volume. Prepare daily.

PROCESS. *Incubation.* Place in an incubation tube, containing 0.1 g. of *70-mesh sand*, a suitable amount of the sample (4 mg. of powdered sweet clover seeds or 4 weighed disks 6 to 7 mm. in diameter cut from the leaves), add 0.1 ml. of water, grind, stopper with the cork carrying the pestle, and incubate for 1 hour at 40°. (Weigh also 4 disks and determine the dry matter.) Add to the incubation tube 4 drops of *ethanol*, grind, then add 1 ml. of *ether*, and again grind.

Filtration. Filter into the side-arm filtration tube through the Büchner funnel fitted with a disk of filter paper and a thick pad of dry asbestos. Again add 4 drops of *ethanol*, grind, add *ether*, grind, and decant, repeating the operation twice more. Wash the pestle, the edge of the test tube, and the funnel with a jet of *ether*. Remove the ether from the filtrate (about 4 ml.) by heating in a water bath at 45°, dilute the residual water and ethanol to the 10-ml. mark with *0.05 N sulfuric acid*, and mix. Warm to about 70°, shake, cool, and filter with suction through a freshly prepared filter, thus obtaining the purified solution.

A. Melilotic Acid. Pipet 4 ml. of the purified. solution into a comparison tube, add 1 ml. of *0.5 N sodium hydroxide solution*, mix, and heat at 85° for 5 minutes. After cooling to room temperature, add 1 ml. of *0.5 N sulfuric acid*, mix, and let stand at least 10 minutes to permit closure of the coumarin ring, then add 1 ml. of *diazonium reagent*, mix, and follow with 0.5 ml. of *5 N sodium hydroxide solution* in small drops with mixing. Dilute to volume and compare immediately with the *standard coumarin solutions*.

B. Melilotic Acid plus Coumarin. Pipet a 1- to 4-ml. aliquot (depending on the cou-

marin content) of the purified solution in a comparison tube and, if less than 4 ml. is taken, add *0.05 N sulfuric acid* to 4 ml. (if not already added), followed by 1 ml. of *7% sodium carbonate solution.* Mix, heat for 5 minutes at 85°, and cool to room temperature. Finally add 1 ml. of *diazonium reagent* dropwise with mixing, dilute to volume, mix, and compare immediately as before.

CALCULATION. Melilotic acid = $A \times 0.9$; coumarin = $B - A$.

2. VOLATILE OILS

As a rule volatile oils are distilled from the raw materials in the region of production. Importers act as middlemen for manufacturers of extracts, also for confectioners, cordial manufacturers, and bakers who use the volatile oil directly in their products, thus eliminating the cost of the alcohol. Only official methods for lemon and orange oils are here given; others appear in the works listed in a footnote under Special Analytical Methods, Spices, above.

Lemon and Orange Oils

Composition. The chief constituent of lemon, orange, and other citrus oils is the terpene *d*-limonene. The chief odorous principle of lemon oil is the aldehyde citral; that of orange oil is decyl aldehyde.

The following data were obtained by Chace [44] on Sicilian oils collected during the years 1907 and 1909 at the factories, largely from bowls of workmen, and by Poore [45] on California oils from a by-products company during 1923 to 1926.

Lemon Oil. Specific gravity at 15.5°, Sicilian 0.8503 to 0.8552, California 0.8529 to 0.8579; refractive index at 20°, Sicilian 1.4740 to 1.4758, California 1.4738 to 1.4749; optical rotation at 20°, Sicilian +54.16 to +66.28, California +52.71 to +70.18; citral (Kleber), Sicilian 3.4 to 5.2, California 2.0 to 3.7, citral (Hiltner), Sicilian 2.6 to 5.3, California 1.4 to 3.7%; esters, California 1.76 to 3.12%.

Orange Oil. Specific gravity at 15.5°, Sicilian 0.8473 to 0.8530, California 0.8467 to 0.8557; refractive index at 20°, Sicilian 1.4723 to 1.4737, California 1.4728 to 1.4748; optical rotation at 20°, Sicilian +92.03 to +99.60, California +94.18 to +99.58; citral (Kleber, calculated as citral, although largely decyl aldehyde), Sicilian, 1.47 to 2.89, California 0.6 to 2.2%; esters, California 0.44 to 2.58%.

Physical and Chemical Values. The following are official methods which were adopted in 1910.[46]

SPECIFIC GRAVITY

Determine at 20°.

REFRACTIVE INDEX

Make readings at 20° with the Abbé or butyrorefractometer.

POLARIZATION

Determine at 20° in a 50-mm. tube, using sodium light. Express results as angular degrees on the 100-mm. basis. Take the reading for orange oil, which falls outside the sugar scale, using a standard levo-reading quartz plate.

TEN PER CENT DISTILLATE

Schimmel & Co. Method.[47] APPARATUS. *A Three-Bulb Ladenburg Flask.* The main bulb is 6 cm. in diameter with a capacity of 200 ml.; the condensing bulbs are 3.5, 3, and 2.5 cm. in diameter; the opening of the side arm is 20 cm. from the bottom of the flask.

PROCESS. Distil 50 ml. of the oil at the rate of 2 cm. per minute until 5 ml. have passed over. Determine the physical constants as directed above.

NOTE. The refractive index of the distillate of both lemon and orange oils is 0.0012 to 0.0022 lower than that of the oil. The optical rotation of the distillate of lemon oil is several degrees lower than that of the oil, but that of the distillate of orange oil is about the same as of the oil.

Chace Nitroso-Chloride Test.[48] PROCESS. To the above 10% distillate, add 5 ml. of *glacial acetic acid* and cool in a freezing bath. Without removing from the bath, add 10 ml. of *ethyl nitrite* and, slowly with constant stirring, 2 ml. of cooled *2 + 1 hydrochloric acid*. Allow to stand in the bath for 15 minutes.

Collect the crystals of pinene nitroso-chloride on a filter and wash with *ethanol*, using suction. Return the filtrate and washings to the freezing bath and after 15 minutes add the crystals to those already on the paper. Wash the joint crops thoroughly with *ethanol*, dry at room temperature, and dissolve in a minimum amount of *chloroform*, then reprecipitate the pinene nitroso-chloride crystals with *methanol*, mount in *olive oil*, and examine under the microscope.

The crystals of pinene nitroso-chloride are broad and have irregular pyramidal ends, whereas those of limonene nitroso-chloride are more or less slender with tuncated ends.

TOTAL ALDEHYDES

Chace Fuchsin Colorimetric Method. Dissolve 2 to 5 g. of the sample in 100 ml. of *aldehyde-free ethanol* and follow the directions below under Extracts.

CITRAL

Kleber Phenylhydrazine Volumetric Method.[49] REAGENT. *Phenylhydrazine Solution.* Redistil the commercial chemical and reject the first portions containing ammonia. Dissolve 10 g. in absolute ethanol and dilute to 100 ml.

PROCESS. Digest 15 g. of the sample for 30 minutes at room temperature with 10 ml. of *phenylhydrazine solution* in a small glass-stoppered bottle. Titrate with *standard 0.5 N hydrochloric acid*, using *methyl orange indicator*.

Blank. Titrate 10 ml. of the reagent in the same manner.

CALCULATION. Subtract the number of milliliters of acid used in the titration of the sample from that used in the titration of the reagent and multiply by 0.076, thus obtaining the weight of citral. In case the end-point is indistinct, add an excess of *standard acid*, transfer to a separatory funnel, draw off the alcoholic liquid, wash the oil with water, and add the washings to the alcoholic solution. Titrate back with *0.5 N sodium hydroxide solution*.

Hiltner Method. Follow the directions given for the Hiltner Method under Extracts, using, however, 2 g. of lemon oil and 8 g. of orange oil diluted to 100 ml., and make the comparison on 2 ml. of this solution.

3. FLAVORING EXTRACTS

a. Almond Extract

Oil of Bitter Almonds. The volatile oil of bitter almonds (not to be confused with the non-volatile or fatty oil) is obtained from the seeds of the bitter almond, apricot, or peach by crushing, addition of water, and distillation. It consists of 90% or over of benzaldehyde ($C_6H_5 \cdot CHO$), which, however, does not exist as such in considerable amount in the seed, but is formed, together with hydrocyanic acid and dextrose, from the glucoside *amygdalin* ($C_{20}H_{27}NO_{11}$) through the action of enzymes in the presence of water.

Formerly a single enzyme, *emulsin*, was thought to bring about the hydrolysis, but Fischer[50] demonstrated that the enzyme *amygdalase* first acts, producing mandelonitrile (prunasin), then another enzyme, *amygdalinase*, completes the reaction. The highly poisonous hydrocyanic acid is removed by various treatments.

When fresh, oil of bitter almonds has usually a specific gravity between the limits 1.044 and 1.066 at 15° and a refractive index of 1.5428 to 1.5439 at 20°, but in exceptional cases exceeds these limits. It is optically inactive or is slightly dextrorotatory. While standing, the oil becomes acid through the formation of benzoic acid. Nitrobenzol, once added as an adulterant, is now rarely if ever present.

Standards. According to the U. S. Standards, almond extract contains not less than 1% by volume of oil of bitter almond free from hydrocyanic acid. Oil of bitter almond, according to the standard, may be obtained from the seeds not only of the bitter almond but also of the apricot and peach.

Benzaldehyde

Denis and Dunbar Phenylhydrazine Gravimetric Method.[51] The principle involved is the precipitation of phenylhydrazone in accordance with the following equation:

$$C_6H_5 \cdot CHO \cdot$$

$$C_6H_5 \cdot CH{:}N \cdot \qquad \cdot H_2O$$

Reagent. *Phenylhydrazine Reagent.* To a mixture of 3 ml. of glacial acetic acid and 40 ml. of water, add 2 ml. of phenylhydrazine. Discard after 1 hour.

Process. Pipet two 10-ml. portions of the sample into 300-ml. Erlenmeyer flasks. Add to one portion 10 ml., to the other 15 ml. of *phenylhydrazine reagent*, shake, stopper tightly, and allow to stand overnight in the dark. Add to each flask 200 ml. of cold water, collect the precipitates on tared Gooch crucibles with thin asbestos mats, wash with cold water, and finally with 10 ml. of *10% ethanol*. Dry either in a vacuum oven (not over 100 mm. of mercury) at 70 to 80° for 3 hours, or in a vacuum desiccator over sulfuric acid, to constant weight.

Calculation. Multiply the weight of the precipitate by 5.408 to obtain the grams of benzaldehyde per 100 ml. of extract. If percentage by weight is desired, divide the grams per 100 ml. by the specific gravity of the extract.

If, owing to an exceptionally high percentage of benzaldehyde, the results on 10 and 15 ml. do not agree, repeat the determination, using a larger quantity of the reagent.

Benzoic Acid

Hortvet and West Benzaldehyde Oxidation Gravimetric Method.[52] The method (Minnesota State Dairy and Food Department) depends on the oxidation of the benzaldehyde to benzoic acid. It was originally designed for the determination of benzaldehyde, but is not suited for that purpose because of the presence of benzoic acid, already formed, derived from the almond oil or formed by oxidation subsequent to the manufacture of the extract. The results serve as a step in the determination of benzoic acid by difference, subtracting from the total benzoic acid the benzoic acid equivalent to the benzaldehyde obtained by the Denis and Dunbar method.

Process. *Oxidation.* Pipet 10 ml. of the sample into a 100-ml. flask, add 10 ml. of *10% sodium hydroxide solution* and 20 ml. of *3% hydrogen peroxide solution*, cover with a watch glass, and heat on a water oven for 20 to 30 minutes or for 5 to 10 minutes after the odor of benzaldehyde has disappeared.

Extraction. Transfer the contents of the flask to a separatory funnel, rinse the watch glass, and add 10 ml. of *1 + 5 sulfuric acid* to

neutralize the sodium hydroxide. Cool to room temperature and shake out with four portions of 25, 25, 20, and 20 ml. of *ether*. Wash the combined extracts with two portions of 5 to 10 ml. of water or until the sulfuric acid is removed as found by testing with *10% barium chloride solution*.

Filter the ether solution of benzoic acid into a tared dish, evaporate at room temperature, dry in a desiccator overnight, and weigh. Multiply the weight by 10 to obtain the weight of total benzoic acid per 100 ml.

CALCULATION. Subtract from the weight of total benzoic acid per 100 ml. the weight of benzoic acid obtained by multiplying by 1.151 the weight of benzaldehyde per 100 ml., obtained by the Denis and Dunbar method. The difference is the weight of benzoic acid per 100 ml. present in the extract.

HYDROCYANIC ACID

Prussian Blue Test. Mix thoroughly a few milliliters of the sample with a few drops of *3% ferrous sulfate solution* and 1 drop of *1% ferric chloride solution*, then add *sodium hydroxide solution* dropwise until no further precipitation takes place. Finally clear with *hydrochloric acid*. The presence of hydrocyanic acid is indicated by the formation of the blue color characteristic of Prussian blue. This test and the following method are tentative.

Vielhaber Silver Nitrate Volumetric Method.[53] PROCESS. Pipet 25 ml. of the sample into a flask, add 5 ml. of a freshly prepared *magnesium hydroxide suspension*, and titrate the alkaline solution with *0.1 N silver nitrate solution*, using *5% potassium chromate solution* as indicator.

CALCULATION. Use the formula: 1 ml. of 0.1 N silver nitrate solution = 0.0027 g. of hydrocyanic acid.

Durien-Smith Alkaline Picrate Colorimetric Method.[54] The method, devised at the Universities of Chicago and Michigan, depends on the reddish brown color of isopurpuric acid formed by a cyanide with an alkaline picrate solution.

APPARATUS. *Colorimeter.*

PROCESS. *Color Formation.* Pipet 3 ml. of *saturated picric acid solution*, 1 ml. of *5% sodium carbonate solution*, and 1 ml. of the solution of the material into a test tube with a graduation mark at 25 ml. Heat in a boiling water bath for 5 minutes, cool under the tap, and make up to the mark.

Color Comparison. Match the color of the solution, set at 20 mm. in a colorimeter, against a *standard 0.002 N potassium or sodium cyanide solution*, treated in the same manner.

NITROBENZENE

The toxicity of nitrobenzene, although much less than that of hydrocyanic acid, renders it a particularly objectionable adulterant of almond oil and extract made therefrom.

Nitrobenzene differs basically from benzaldehyde in that it contains about 11% of nitrogen, but in an extract containing only 1% of volatile oil this would be scarcely determinable.

Tentative A.O.A.C. Test. Boil a few milliliters of the extract with *zinc dust* and *acetic acid*. Filter and heat the filtrate with a drop of *chloroform* and an excess of *10% sodium hydroxide solution*. The presence of nitrobenzene is indicated by the formation of the odor of phenylisonitrile.

Bisulfite Test.[55] Shake vigorously 20 ml. of the extract in a test tube with 5 to 10 ml. of a cold saturated aqueous solution of *sodium bisulfite*. Transfer to an evaporating dish and heat until dealcoholized. Benzaldehyde remains in the hot solution as a crystalline salt and no odor of almond oil is apparent, whereas nitrobenzene forms oily drops on the surface of the hot liquid and is further recognized by its pungent odor.

b. Cinnamon, Cassia, and Clove Extracts

Standards. All three extracts, according to the U. S. Standards, must contain 2% by volume of the respective oils.

Volatile Oil

Hortvet and West Wet Ether Extraction Gravimetric Method.[56] The method was devised at the Minnesota Food and Drug Laboratory.

Process. Pipet 10 ml. of the sample into a separatory funnel, dilute with 50 ml. of water, acidify with *1 + 1 hydrochloric acid*, and shake out the oil with three successive portions of *ether* measuring 50, 30, and 20 ml. respectively. Wash the combined ether solutions in another separatory funnel with two portions of about 30 ml. each of water. Dry the ether solution of cinnamon and cassia extracts by shaking with *granular calcium chloride* (or, better, *anhydrous sodium sulfate*). Filter through a dry paper into a tared widemouth flask, rinsing the separatory funnel and washing with *ether*. Evaporate the ether rapidly on a boiling water bath until only a few drops remain, then remove the flask and while still hot rotate so as to distribute the oil over the inner surface and pour out the ether vapor. Dry the outer surface and weigh after allowing to stand on the balance pan 2 or 3 minutes for the film of moisture to disappear.

Calculation. Obtain the volume of oil corresponding to the weight by dividing by 1.05 for cassia, 1.03 for cinnamon, and 1.055 for cloves, then multiply by 100 and divide by 10 to obtain the percentage by volume.

Tests. Dissolve a portion of the oil in *ethanol* and add a drop of *ferric chloride solution*. The coloration with the cinnamon oil is green, with cassia oil brown, and with clove oil deep blue. The refractive indices of the three oils are: cinnamon 1.590 to 1.599, cassia 1.585 to 1.600, and cloves 1.560 to 1.565.

Cinnamic Aldehyde

See Spices, under Cassia above.

c. Ginger Extract

Standard. The U. S. Standards specify that ginger extract must contain in each 100 ml. the alcohol-soluble matter from not less than 20 g. of ginger.

Methods. No satisfactory quantitative methods have been devised for the determination of the valuable constituents of ginger extract. The following qualitative tests are for ginger extractives and capsicum or allied pungent principles.

Ginger Extractives

Seeker Acid Vanillin Test.[57] To 10 ml. of the sample, add 20 ml. of water and dealcoholize by evaporation to 20 ml. Shake in a separatory funnel with an equal volume of *ether*, transfer the ether solution to a porcelain dish, and allow to evaporate spontaneously. To the residue, add 5 ml. of *75% sulfuric acid* and 5 mg. of *vanillin*. After allowing to stand 15 minutes, add an equal volume of water. The presence of ginger extractives is indicated by the formation of a blue color.

Capsicum

Nelson Test Modified by LaWall and Doyle.[58] Process. *Ether Extraction.* To 10 ml. of the extract, add *1 + 9 sodium hydroxide solution* to slight alkaline reaction as indicated by *litmus paper*. Evaporate at 70° to one-quarter the original volume and acidify slightly with *1 + 9 sulfuric acid*. Extract in a separatory funnel with an equal volume of *ether*, shaking gently 1 to 2 minutes to avoid an emulsion. Remove the aqueous layer, wash the ether layer once with 10 ml. of water, and transfer to a small evaporating dish.

Saponification. Add *0.5 N ethanolic potassium hydroxide solution* to a distinctly alkaline reaction, evaporate at 70° to a pasty condition, then add 20 ml. more of the *alkali solution* and heat for 30 minutes on the steam bath or until the gingerol is completely saponified.

Second Extraction. Transfer the residue to a small separatory funnel with 50 ml. of water and extract with 50 ml. of *ether*. Wash the ether extract repeatedly with small portions of water until neutral, transfer to a small evaporating dish, and allow to evaporate spontaneously.

Test. Apply the finger to the residue and taste. A persistent stinging sensation indicates capsicum or other foreign substance.

d. Lemon Extract

Contrary to the popular belief, lemon extract is not made from the pulp of the lemon but solely from the lemon peel, either directly by maceration with ethanol or by simple solution in ethanol of the volatile oil derived from the peel.

Standards. The U. S. Pharmacopoeia directs to macerate 500 g. of the peel from the fresh fruit with 95% alcohol and make up to 1 liter.

The U. S. Standards permit the use of either lemon peel or lemon oil or both in *lemon extract*, but require a content of at least 5% by volume of the oil. *Terpeneless lemon extract* may be prepared by shaking lemon oil with dilute alcohol, or by dissolving terpeneless lemon oil in dilute alcohol, but it must contain not less than 0.2% by weight of citral derived from lemon oil.

Volatile Oil

Mitchell Polarization Method.[59] When the extract is a tincture without other addition, polarize directly in a 200-mm. tube at 20°. If sugar or other optically active foreign constituents are present, dealcoholize a definite volume, shake out with *ether* until the volatile oil is removed, dilute the aqueous liquid to the original volume, polarize, and correct the original polarization accordingly.

To obtain the per cent by volume of oil, divide the reading (corrected if necessary) on the Ventzke scale by 3.4 (Mitchell) or 3.2 (A.O.A.C.).

Mitchell Centrifugal Method.[60] Process. Mix in a Babcock milk test bottle 20 ml. of the sample, 1 ml. of *1 + 1 hydrochloric acid*, and 25 to 28 ml. of water warmed to 60°. Mix, keep for 5 minutes in a bath containing water at 60°, then whirl in a centrifuge for 5 minutes. Add water at 60° sufficient to bring the oil into the graduated neck and whirl for 2 minutes. Place again in water at 60° for a short time, read the top and bottom meniscus of the oil column, and subtract.

Determine the refraction of the oil. The limits for pure lemon oil are given under the oil above.

Calculation. If the percentage as read is over 2.0%, add 0.4%, if less than 2.0% but over 1.0%, add 0.3%, thus obtaining the corrected per cent by volume.

If the percentage by weight is desired, multiply the percentage by volume by either method by 0.86 and divide the product by the specific gravity of the original extract.

Note. Boyles[61] recommends to dilute 10 ml. of a non-alcoholic extract to the mark in a 50-ml. volumetric flask with *ethanol*, mix thoroughly, allow to stand 30 minutes, and filter through a dry paper; then proceed with 20 ml. as with an alcoholic extract, multiplying by 5 to correct for the dilution.

Total Aldehydes

Chace Fuchsin Colorimetric Method.[62] Reagents. *Aldehyde-Free Ethanol.* Digest for 24 hours at room temperature with frequent shaking 5 g. of *m*-phenylenediamine hydrochloride with 1 liter of ethanol. Reflux

for at least 8 hours, allow to stand overnight, and distil. Reject the first 10 and the last 5 ml. of the distillate. Store in a dark, cool place in well-filled bottles. Test 25 ml. by digestion for 20 minutes in the cooling bath with 20 ml. of fuchsin solution. If a color deeper than faint pink forms, repeat the treatment with *m*-phenylenediamine hydrochloride.

Fuchsin Solution. Dissolve 0.5 g. of fuchsin in 250 ml. of water, add an aqueous solution of SO_2 containing 16 g. of the gas, and allow to stand until colorless, then make up to 1 liter with distilled water. Store in a refrigerator and use only after 12 hours. Reject after 3 days.

Standard Citral Solution. Dissolve 0.1 g. of citral in 100 ml. of 50% by volume aldehyde-free ethanol. Reject after keeping 3 or 4 days; 1 ml. = 1 mg. of citral.

PROCESS. Pipet 25 ml. of the sample into a tared 50-ml. volumetric flask and weigh flask and extract. Make up to the mark at room temperature with *aldehyde-free ethanol* and shake. Pipet 2 ml. into the tube of a colorimeter or a color comparison tube, add 25 ml. of *aldehyde-free ethanol* and 20 ml. of *fuchsin solution*, both cooled to 14 to 16°, and complete the volume of 50 ml. with *aldehyde-free ethanol.* Shake well, stopper, and place in a cooling bath at 14 to 16° for 15 minutes.

Blank. Place also in the bath at the same time a tube containing a mixture of 2 ml. of *standard citral solution* and the same amount of *reagents* as the unknown.

Color Comparision. Compare the color of the known and unknown immediately after removal from the heat. Calculate the amount of aldehydes in terms of citral present in the unknown and repeat the procedure, using a quantity of *standard citral solution* corresponding approximately to the amount derived from the sample.

CALCULATION. From the latter color comparison, calculate the exact amount present in the sample.

It is essential that both tubes (or all the tubes if a series of tubes of the known are prepared containing 1 to 4 mg. in 0.5-mg. increments) and reagents be kept at the same temperature (14 to 16°) and that the comparisons be made within 1 minute after removal from the bath.

CITRAL

Hiltner *m*-Phenylenediamine Colorimetric Method.[63] REAGENTS. *m-Phenylenediamine Hydrochloride Solution.* Dissolve 1 g. of the substance in 45 ml. of 85% by volume ethanol and 1 g. of crystalline oxalic acid in the same amount of ethanol of the same strength. Pour the two solutions into a 100-ml. volumetric flask, add 2 to 3 g. of fuller's earth or animal charcoal, make up to the mark, shake, and filter through a double paper. The solution, after this treatment, should be clear and colorless. Prepare fresh each day or decolorize an old solution by shaking again with fuller's earth.

Ethanol. Clear colorless ethanol, full strength for ordinary alcoholic extracts, diluted with an equal volume of water for terpeneless extracts. If colored, add NaOH to slight alkaline reaction and distil. Removal of aldehydes is not necessary.

Standard Citral Solution. See Chace Method above.

PROCESS. Pipet 25 ml. of the sample into a tared 50-ml. volumetric flask, weigh, and make up to the mark with *ethanol* if an alcoholic extract, or *half-strength ethanol* if a terpeneless extract, all at room temperature. Stopper and shake.

Preliminary Comparison. Pipet 2 ml. of the diluted extract into the colorimeter or comparison tube, add 10 ml. of *m-phenylenediamine hydrochloride solution*, and make up to 50 ml. Compare with a mixture of 2 ml. of the *standard citral solution* treated with the reagent in like manner.

Final Comparison. Calculate from the ap-

proximate result of the preliminary comparison the amount of standard citral solution required for a perfect match with the unknown; make a final comparison using that amount.

Bailey and Beebe Photoelectric Modification.[64] In adapting the Hiltner method to measurement in the Klett-Summerson photoelectric colorimeter, Bailey and Beebe (Illinois Department of Agriculture) proceed as follows.

APPARATUS. *Klett-Summerson Photoelectric Colorimeter*, with a blue 420-mμ light filter.

REAGENT. *Phenylenediamine Reagent.* Dissolve 1 g. of *m*-phenylenediamine hydrochloride and 1 g. of oxalic acid each in about 45 ml. of 85% ethanol. Pour the two into a 100-ml. volumetric flask, add 2.5 g. of fuller's earth, dilute to the mark with 85% ethanol, mix well, and filter while the fuller's earth is in suspension, pouring the first 15- to 20-ml. portions back on the filter. The solution is stable for 2 days.

PROCESS. *Color Formation.* Transfer 10 g. of the sample with *ethanol* to a 100-ml. volumetric flask, using *95% ethanol* for lemon extract and *50 to 95% ethanol* for terpeneless extracts. Pipet exactly 10 ml. of the *phenylenediamine reagent* into the solution and complete the volume with ethanol.

If the sample is not clear and colorless, run a sample blank determination in another 100-ml. flask, using 10 g. of the sample and 10 ml. of *1% oxalic acid solution in 85% ethanol*, otherwise no blank is necessary. Also run a reagent blank in the same manner as the actual determination, omitting the sample and completing the volume with *50% ethanol.*

Centrifuge a sufficient quantity of emulsion-type extracts in a 20-ml. tube after completing the volume to 100 ml. until a clear solution is obtained and run a blank in like manner. Run a blank also if the sample is cloudy even though colorless.

Color Reading. Use a blue 420-mμ light filter and read the logarithmic scale.

CALCULATION. Obtain the percentage of citral (C), by the formula

$$C =$$

in which F is the factor and U, R, and S are the readings of the unknown, the reagent blank, and the sample blank respectively.

Derive the factor (F) as follows. Prepare a 1% solution of pure citral in ethanol. Pipet 1 or 2 ml. into a 100-ml. volumetric flask, add 10 ml. of the phenylenediamine reagent, and dilute to the mark with ethanol. The solution contains 0.01 or 0.02 g. per 100 ml., equivalent respectively to 0.1 or 0.2% of an unknown diluted ten-fold.

Prepare a series of solutions and run a reagent blank. Calculate the factor by the equation given above. For example, if the citral solution contains 0.20% (C) of citral, the scale reading (U) is 228 and $U - R$ is 213, S being 0, then the factor (F) is 0.000939. The average factor for percentages 0.00 to 0.20 as determined by Bailey and Beebe is 0.000953.

ETHANOL

Approximate Method. If sugar, glycerol, or other foreign water-soluble constituents are absent, determine the specific gravity at 20° by means of a pycnometer or Westphal balance and find the corresponding per cent by volume from the table in Part II, F1. Deduct the per cent of lemon oil as determined above.

Official Exact Method. Pipet 50 ml. of the sample into a 200-ml. volumetric flask, mix, and make up to the mark the aqueous liquid below the separated oil, then transfer to a larger flask. Shake with 5 g. of *magnesium carbonate,* filter through a dry paper, and distil an aliquot of the filtrate as directed in Part II, F1.

Methanol

See also Part II, F3.

Leach and Lythgoe Method. In addition to weighing and determining the apparent percentage of ethanol in the distillate, as directed above, determine the refraction with the immersion refractometer and proceed according to the Leach and Lythgoe Method as directed in Part II, F3.

Mulliken and Scudder Test.[65] Convert methanol into formaldehyde by plunging a spiral of copper wire, heated to redness in the oxidation flame, into a test tube containing a portion of the distillate, repeating for several times.

Test for formaldehyde as directed in Part I, C13.

Colors

Albrech Test for Peel Color.[66] To several milliliters of the sample contained in a test tube, add 3 to 4 volumes of *hydrochloric acid;* to another portion, add a few drops of *ammonium hydroxide*. A marked deepening of the color in both tests indicates color derived from the peel.

Methods for Artificial Colors. See Part I, C12. Note especially the color changes with acid (red for colors of the tropeolin group, partial decolorization for naphthol yellow S, and complete decolorization for naphthol yellow and dinitrocresol). Test for turmeric with boric acid.

e. Maple Flavor

Choline

Extract of fenugreek, which is the chief flavoring constituent of certain imitation maple flavors, contains choline which may be isolated and weighed as reineckate, then subjected to microscopic and physical examination.

Wilson and Keenan Reineckate Gravimetric Method.[67] Reagent. *Reineckate Reagent.* Place 0.4 g. of $NH_4[Cr(NH_3)_2(SCN)_4]\cdot H_2O$ (ammonium reineckate) in a small Erlenmeyer flask, add 15 ml. of water, shake 2 to 3 minutes, and filter. The reagent must be prepared just before use.

Process. *Choline Reineckate Precipitation.* To 25 ml. of the sample in a 50-ml. beaker, add 5 ml. of *reineckate reagent,* cool in an ice water bath with stirring for 1 to 2 minutes, and allow to remain in the bath for about 30 minutes longer, stirring several times for about 30 seconds each. Filter through a thin mat of asbestos in a Gooch crucible, rinsing the beaker with two or three 5-ml. portions of water, then similarly with *ethanol,* followed by *ether*.

Acetone Treatment. Transfer the crucible to another suction assembly provided with a small test tube to catch the washings, rinse the beaker and crucible with 2 or 3 ml. of *acetone,* repeating the washings until the liquid coming through is no longer pink. Transfer the acetone solution to a weighed 10-ml. beaker and evaporate to dryness in an air current. Rinse the beaker and crucible with 1 or 2 ml. of *acetone* and evaporate to dryness in a current of air. Wash down any crystals with 1 or 2 ml. of *acetone* and again evaporate. Dry in a vacuum desiccator over sulfuric acid for several hours to remove the last traces of acetone and weigh.

Calculation. Convert the weight of reineckate into weight of choline by the factor 0.287.

Identification. Dissolve 0.65 g. of *ammonium reineckate* in 20 ml. of water and filter into 10 ml. of water in which has been dissolved 0.2 g. of *choline chloride*. Use 15 ml. of water to rinse the beaker and wash the precipitate. A precipitate forms which, on being stirred for a few minutes, becomes silvery pink. Filter on a fritted-glass funnel and wash once with 10 ml. of water. Compare the micro crystallographic properties of this

preparation with that obtained in the analysis, which, however, has the fenugreek odor not present in the synthetic product.

f. Nutmeg Extract

Standards. According to the U. S. Standards, nutmeg extract must contain at least 2% of oil of nutmeg.

VOLATILE OIL

Mitchell Centrifugal Method. See Lemon Extract above.

g. Orange Extract

Orange oil, terpeneless orange oil, orange extract, and terpeneless orange extract are analogous to lemon oil and lemon extract.

Standard. According to the U. S. Standards, orange oil has an optical rotation of at least +95° in a 100-mm. tube at 20° and orange extract contains 5% by volume of orange oil. No requirement for citral in terpeneless orange extract is specified, since the aldehyde is nearly, if not all, decyl aldehyde, but it is specified that it correspond in flavoring strength to orange extract, meaning, it is inferred, the aldehyde strength.

Composition. The composition of orange oil and its content of decyl aldehyde, instead of citral, are given above under Volatile Oils.

Methods. The methods are the same as given under Lemon Extract above, but the percentage by volume of orange oil is obtained from the polarization at 15° by dividing by 5.3 (Mitchell) or 5.2 (A.O.A.C.) and the percentage by weight by either method by multiplying the percentage by volume by 0.85 and dividing by the specific gravity of the extract.

h. Peppermint and Spearmint Extracts

Standards. According to the U. S. Standards, both peppermint and spearmint extracts must contain 3% by volume of the respective oils.

VOLATILE OIL

Mitchell-Howard Centrifugal Method.[68] PROCESS. Pipet into a Babcock milk test bottle 10 ml. of the sample, then add 25 ml. of water, 1 ml. of *hydrochloric acid,* and 0.5 ml. of *chloroform.* Stopper the bottle or close with the thumb and shake vigorously for at least 1 minute. Centrifuge for 2 minutes, thus forcing the volatile oil and chloroform to the bottom of the bottle. Draw off the supernatant liquid, except 3 or 4 ml., by means of a slender tube connected with an aspirator.

Add to the residue 1 ml. of *ether,* shake well, and rotate the bottle for 1 minute in boiling water, holding at an angle and keeping immersed up to the neck, thus sweeping out all the chloroform with the ether without appreciable loss of oil. Cool, fill the bottle to the top of the neck with *saturated salt solution* at room temperature, centrifuge for 30 seconds, and read the volume of the oil column to the top meniscus.

CALCULATION. Multiply by 2 to obtain the percentage by volume. To convert percentage by volume into percentage by weight, multiply by 0.9 and divide by the specific gravity of the extract.

NOTE. The Tentative A.O.A.C. Method for peppermint, spearmint, and wintergreen is much like the Howard method, but (1) *carbon bisulfide* is substituted for chloroform, (2) no ether is added, (3) the time of the first and last centrifuging is increased to 6 and 2 minutes respectively, and (4) the aspiration is carried out with the bath at 70° for 3 minutes, shaking every 15 seconds, then with the bath at boiling for 45 seconds.

MENTHOL

Brignall Acetylation Volumetric Method.[69] The following method is proposed by Brig-

nall (A. M. Todd Company, Kalamazoo, Mich.) to correct the well-known errors of the Power and Kleber method.[70] It may also be applied to the oil separated from the extract.

Reagent. *Acetylant Mixture.* Four parts by volume of *n*-butyl ether and one part of $(CH_3 \cdot CO)_2O$.

Process. *Acetylation.* Weigh 1 g. of the sample into a tared acetylation flask, mix, and treat with exactly 5 ml., measured by a Koch micro buret, of a freshly prepared *acetylant mixture.* Prepare a blank in an identical manner, omitting the oil. Connect the flasks to air condensers and boil gently for 1 hour on a sand bath. Without removing the flasks from the bath, add 20 ml. of hot distilled water through the condensers and boil the contents vigorously for an additional 30 minutes to convert the excess anhydride into acetic acid. Remove the flasks from the bath, allow to cool to room temperature, and add 20 ml. of cold distilled water.

Titration. Remove the flasks from the condensers and rinse the connections, allowing the rinsings to flow into the flasks. At this point the acid strength of the blank is approximately 0.5 *N*. Add 8 to 10 drops of *1% ethanolic phenolphthalein solution,* and neutralize the excess acid with *0.5 N ethanolic potassium hydroxide solution.* Titrate the oil sample to the full red color of the indicator and match the oil sample with the blank. Although it is necessary to conduct the titration on a 2-phase system, this presents no difficulty.

Calculation. Obtain the difference (K) in the volumes of standard 0.5 *N* potassium hydroxide solution, then employ the following formula for calculation of the percentage of free alcohol (P):

in which M is the molecular weight of the alcohol divided by 20 and G is the weight of the sample.

If the percentage of total alcohols (P') is desired, make an ester determination by the regular method and employ the following formula:

$$\frac{L}{M''} \qquad P''$$

in which M' is the molecular weight of the alcohol and M'' and P'' are the molecular weight and per cent respectively of the esters.

i. Rose Extract

Standards. According to the U. S. Standards, rose extract must contain at least 0.4% of otto of roses.

Volatile Oil

Hortvet and West Wet Ether Extraction Method.[71] Process. Mix in a separatory funnel 20 ml. of the sample and 50 ml. of water, then add 1 ml. of *1 + 1 hydrochloric acid* and extract with three portions of 20 ml. each of *ether.* Transfer the combined ether solutions to a 150-ml. flask and shake for a few minutes with *granulated calcium chloride.* Allow to settle and filter through a dry paper into a tared covered dish. Wash the calcium chloride and filter twice with 10 ml. each of *ether,* allow the ether to evaporate at room temperature, then dry to constant weight in a vacuum desiccator over sulfuric acid.

Calculation. Divide the final weight by 0.86 and multiply by 5 to obtain the percentage by volume of rose oil.

j. Vanilla Extract and Its Substitutes

Various methods are in use for the preparation of vanilla extract, but in the United States it is commonly recognized that 1 liter should contain the flavoring constituents of 100 g. of vanilla beans removed by a mixture of 650 parts of ethanol and 350 parts of water

and should not contain synthetic vanillin or coumarin, or tonka bean extract. If prepared according to the U. S. Pharmacopoeia of 1907, 1 liter contains 200 g. of sucrose. The sucrose may be omitted or glycerin substituted for it in the preparation of the extract without materially changing the content of vanillin.

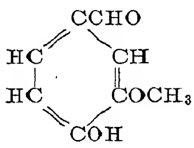
The vanilla bean is not a true bean, but a slender, much elongated fruit, containing numerous black seeds, of a tropical orchid *Vanilla planifolia* Andrew.

Vanillin (methylprotocatechuic aldehyde), the chief flavoring constituent of the vanilla bean, is a derivative of vanillic acid. It crystallizes as narrow, colorless prisms or needles melting at 80 to 81°.

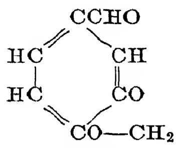
Related to vanillin is heliotropin or piperonal (dioxymethylene protocatechuic aldehyde) which, although delightfully fragrant, is seldom used as a food flavor.

The tonka bean is the fleshy seed of a legume, *Coumarouna odorata* Aubl., and is consequently entitled to be called a bean. Coumarin, its flavoring constituent, is the anhydride of coumaric acid which forms colorless crystalline scales melting at 67°.

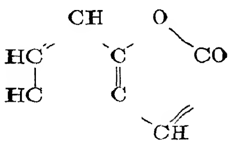
Imitation vanilla extract consists usually of a solution of synthetic vanillin, with or without synthetic coumarin or tonka bean extract, colored with caramel.



VANILLIN



HELIOTROPIN



COUMARIN

Chemical Composition. Following is a summary of analyses by Winton, Berry, and Albright [72] of 71 vanilla extracts made in the laboratory as above described from various commercial grades and lengths of beans, also the average of two analyses of samples of tonka bean extract. The extracts made from Mexican beans, the highest grade, contained 0.15 to 0.20% of vanillin, those from Tahiti beans, the lowest grade, 0.11%. The commercial value, however, is not determined by vanillin content. Extracts made from Comores beans contained as high as 0.31% and extracts made from Seychelles, Madagascar, Bourbon, South American, and Javanese beans contained maximums of 0.21 to 0.30%.

From these figures it is obvious that constituents other than vanillin constitute the flavor and it is because of the absence of these constituents that a solution of synthetic vanilla is not acceptable to the connoisseur. Although prices fluctuate, the flavoring constituents of an imitation extract may cost only one-tenth or even one-twentieth that of a genuine extract.

The authors named found that reducing the strength of the ethanol in the menstruum or addition of glycerol or sugar did not greatly influence the vanillin content of the extract, but in some cases changed the lead number or the color values. Dean and Schlotterbeck [73] report a marked increase in these values by adding potassium carbonate up to 0.4%.

SOLIDS

Steam Oven Gravimetric Method. Evaporate 1 to 2 g. of the extract to dryness in a flat-bottom dish, 5 cm. in diameter, and dry to practically constant weight in a steam or boiling water oven, or until the loss is due to the slow evaporation of vanillin and coumarin, which for practical purposes may be ignored. For example, in an extract containing 20% of solids due to sucrose, the error

Composition of Authentic Vanilla and Tonka Extracts

	Vanillin g. per 100 Ml.	Normal Lead No.	Color of Extract			Color of Lead Filtrate *					Amyl Alcohol Insol. Color
			R	Y	R : Y	R	Y	R : Y	R	Y	
					1 :			1 :	%	%	%
Vanilla extract											
Min.	0.11	0.40	15	40	2.3	0.6	3.1	2.8	4	5	16.0
Max.	0.31	0.74	56	177	3.9	3.4	14.6	5.8	8	10	35.7
Aver.	**0.19**	**0.54**	**32**	**102**	**3.2**	**1.8**	**7.6**	**4.2**	**6**	**8**	**25.5**
Tonka extract											
Aver.	....	**0.11**	**5**	**19**	**3.7**	**0.5**	**2.4**	**4.8**	**10**	**13**	**30.8**

	Acidity †			Ash: g. per 100 ml.			Alkalinity of Ash ‡		
	Total	Of vanillin	Not vanillin	Total	H_2O sol.	H_2O insol.	Total	H_2O sol.	H_2O insol.
Vanilla extract									
Min.	30	7	14	0.220	0.179	0.027	30	22	7
Max.	52	20	42	0.432	0.357	0.081	54	40	18
Aver.	**42**	**12**	**30**	**0.319**	**0.265**	**0.054**	**42**	**30**	**12**
Tonka extract									
Aver.	**5**	..	**5**	**0.147**	**0.139**	**0.008**	**15**	**13**	**2**

* Calculated to volume of extract. † Milliliters of 0.1 *N* alkali per 100 ml. ‡ Milliliters of 0.1 *N* acid per 100 ml.

due to the full amount of 0.20% of vanillin is relatively insignificant.

Vanillin and Coumarin

Hess and Prescott Ether-Ammonia Gravimetric Method.[74] The underlying principle is the formation by ammonium hydroxide, acting on an ether solution of vanillin and coumarin, of a water-soluble compound of vanillin, whereas coumarin remains unchanged.

The following description of the method differs from the original method in certain details of manipulation, introduced by Winton in collaboration with Silverman, Bailey, and Lott,[75] whereby the presence of acetaldehyde, at one time much used as an adulterant of synthetic vanillin, may be detected and normal lead number and color value of the lead filtrate may be determined in the same weighed portion.

Reagent. *Standard Lead Acetate Solution.* Dissolve 80 g. of crystalline $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ in water and make up to 1 liter.

Process. *Dealcoholization.* To a 250-ml. beaker with marks showing volumes of 80 and 50 ml., add 50 g. of the extract or imitation of the usual dilution, or a smaller amount if either vanillin or coumarin is present in excessive amount. Dilute to 80 ml. and evaporate to 50 ml. in a water bath held at 70°. Repeat the dilution and evaporation, then transfer to a 100-ml. volumetric flask and rinse with hot water.

Lead Precipitation. Add 25 ml. of *standard lead acetate solution,* make up to the mark with water boiled until carbon dioxide is removed, shake, and allow to stand 18 hours in a water bath or bacteriological incubator

kept at 37 to 40°. Filter through a small dry paper and pipet 50 ml. of the filtrate into a separatory funnel for the determination of vanillin and coumarin and 10 ml. into a beaker for the determination of lead number and color value of the lead filtrate.

The adjustable multiple stand devised by one of us (Fig. 203) permits ready introduction and removal of the funnels, and holds them firmly in place while drawing the liquid from one to another.

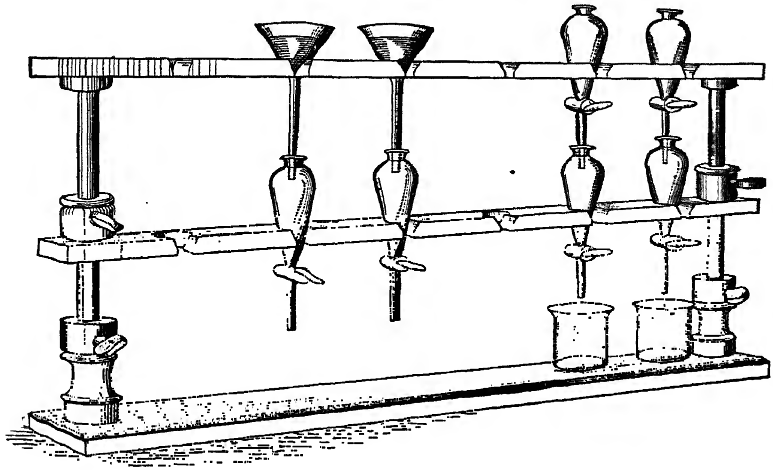


FIG. 203. Multiple Separatory Funnel Support.

Ether Extraction. To the 50-ml. aliquot in the separatory funnel, add 20 ml. of *ether*, shake well, and draw off the aqueous liquid and any ether emulsion into a beaker, then remove the clear ether solution to a second separatory funnel. Return the aqueous solution and emulsion to the first separatory funnel and shake out three times, using, however, portions of only 15 ml. of *ether*.

Shake the combined ether solutions with 10 ml. of *2% ammonium hydroxide* and repeat three or four times, but using 3 ml. of *2% ammonium hydroxide*, taking care that none of the ether solution passes through the cock. Reserve the ether solution for the determination of coumarin.

VANILLIN. Add to the ammoniacal solution *10% hydrochloric acid* to acid reaction. Do this without delay, since on standing the solution darkens with loss of vanillin. Cool, shake out in a separatory funnel with four portions of *ether* as in the first ether extraction. Evaporate the combined ether solutions at room temperature and dry in a sulfuric acid desiccator to constant weight. If pure vanillin, the residue will have a melting point of 80°.

COUMARIN. Draw off the ether solution which remains after shaking out the vanillin with ammonium hydroxide, into a tared dish, allow the ether to evaporate at room temperature, dry in a sulfuric acid desiccator to constant weight. The residue, if pure coumarin, will have a melting point of 67° and be completely soluble in 3 or 4 portions of *petroleum ether*. It will respond to the Leach test.

If a residue remains after treatment with petroleum ether, dry over sulfuric acid and

determine the melting point. Acetaldehyde melts at 112°.

CALCULATION. Multiply the weight of vanillin and coumarin by 4 to obtain the percentages in the extract.

VANILLIN

Folin and Denis Phosphotungstic-Phosphomolybdic Colorimetric Method.[76] Results by this method (Harvard Medical School) agree closely with those by the Hess and Prescott method.

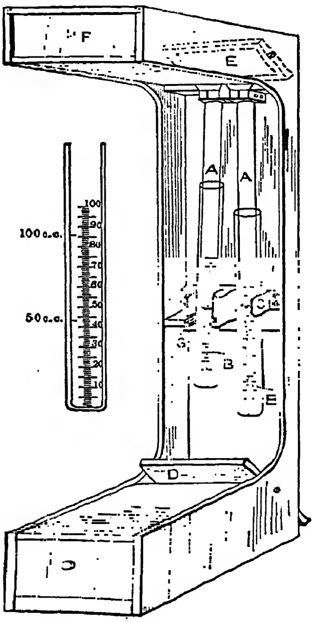


FIG. 204. Schreiner Colorimeter.

APPARATUS. *Colorimeter* or *Nessler Tubes.*

REAGENTS. *Phosphotungstic-Phosphomolybdic Reagent.*[77] Boil for 1.5 to 2 hours a mixture of 100 g. of pure $Na_2WO_4 \cdot 2H_2O$, 20 g. of nitrate- and ammonia-free phosphomolybdic acid, 100 g. of sirupy 85% H_3PO_4, and 700 ml. of water. Cool, filter, and make up to 1 liter.

Lead Acetate Solution. Prepare a solution of 50 g. each of lead acetate and basic lead acetate in water and dilute to 1 liter.

PROCESS. *Lead Precipitation.* Pipet 5 ml. of the sample into a 100-ml. volumetric flask, add about 75 ml. of cold water, then 4 ml. of *lead acetate solution.* Make up to the mark, shake, filter rapidly through a dry pleated paper, and pipet 5 ml. (= 0.25 ml. of the extract) into a 50-ml. volumetric flask.

Into a second 50-ml. volumetric flask, pipet 5 ml. of *0.01% standard aqueous vanillin*

Color Formation. To each solution, add from a pipet 5 ml. of the *phosphotungstic-phosphomolybdic reagent,* moving the pipet so as to rinse down the vanillin solution. Gently rotate to mix the liquids, allow to stand 5 minutes, and fill to the mark with *saturated sodium carbonate solution.* Invert several times to effect thorough mixing, allow to stand 10 minutes to complete the precipitation, and filter rapidly through a dry pleated paper.

Color Comparison. Make the color comparison of the clear blue liquids in a colorimeter with the standard set at 20 mm. or in Nessler tubes. The Schreiner Colorimeter (Fig. 204) is inexpensive and well suited for the purpose.

CALCULATION. Obtain the grams of vanillin per 100 ml. (G) of the extract as follows:

$$G = \frac{100 \times 0.0005 \times K \times 100}{5 \times 5 \times U} = \frac{0.2K}{U}$$

in which K and U are the readings of the known and unknown respectively.

Estes Acid Mercuric Nitrate Colorimetric Method.[78] The little known process is simple and employs only one active reagent.

APPARATUS. *Colorimeter* or *Nessler Tubes.*

REAGENT. *Acid Mercuric Nitrate Solution.* Dissolve 10 g. of metallic mercury in 20 g. of HNO_3 (sp.gr. 1.42) and dilute with 250 ml. of water.

PROCESS. Pipet 5 ml. of the sample into a 50-ml. volumetric flask, dilute with 6 ml. of water, and add 1.5 ml. (1 ml. for non-alcoholic extracts) of the *acid mercuric nitrate solution*.

At the same time pipet into a 50-ml. volumetric flask 5 ml. of *0.1% standard aqueous vanillin solution*, dilute with 6 ml. of water, and add 0.5 ml. of the *reagent*.

Digest both solutions in a boiling water bath for 20 minutes to bring out the violet or violet-red color, cool rapidly, make up to the mark, filter, and compare in a colorimeter or Nessler tubes.

Dox and Plaisance Thiobarbituric Acid Gravimetric Method.[79] The method is applicable only in the absence of caramel, as shown by the phloroglucinol test applied in the course of the determination.

REAGENTS. *Thiobarbituric Acid Reagent.* Dissolve the acid in 12% HCl.

Phloroglucinol Solution. See Part I, C6*a*, Pentoses and Pentosans.

PROCESS. *Test for Caramel.* Dealcoholize and clarify with *lead acetate* as in the Hess and Prescott method, except that only 25 ml. of the extract are used which, after dealcoholizing, are transferred to a 50-ml. flask. Test a small portion of the lead filtrate with *phloroglucinol solution*. If caramel is present, a brown precipitate appears; if absent, the vanillin gives a rose-pink color or slightly pink precipitate.

Thiobarbituric Acid Precipitation. In the absence of caramel, transfer 40 ml. of the filtrate to another 50-ml. flask, add *hydrochloric acid* to the mark, and after allowing to stand a few minutes filter from the lead chloride through a dry paper. To 40 ml. of the filtrate, add *thiobarbituric acid reagent* in excess, allow to stand overnight, collect the orange-colored precipitate on a Gooch crucible, wash with 50 ml. of *12% hydrochloric acid* in small portions and finally with 20 ml. of water, dry at 98°, and weigh.

CALCULATION. Correct the weight of the precipitate for solubility by adding 0.0026 g. and calculate the percentage of vanillin (P) from the weight of the precipitate (W) by the following formula:

$$\frac{50 \times 50 \times 0.5462W}{40 \times 40 \times 25} = 0.0341\,W$$

Daniels, Emery, and Prather Iodoxy Ammonium Benzoate Colorimetric Method.[80] In this method (College of Pharmacy of the University of California) photoelectric measurement is employed.

APPARATUS. *Photometer* with 5-mm. cell and filter S 43.

REAGENTS. *Iodoxy Ammonium Benzoate Solution,* 0.5%. Dissolve 1 g. of *o*-iodoxy ammonium benzoate in 200 ml. of water.

Hydrochloric Acid, 0.167 N (constant boiling).

PROCESS. *Lead Precipitation.* Pipet 10 ml. of vanilla extract into a 50-ml. volumetric flask, add 25 ml. of water and 5 ml. of *8% lead acetate solution*, then make up to the mark with water and filter through a dry paper.

Color Formation. Pipet 5 ml. of the lead filtrate (or 2 ml. of the lead filtrate prepared for the gravimetric method for the determination of vanillin, lead number, etc.) into a 50-ml. volumetric flask, add 25 ml. of *0.167 N hydrochloric acid*, shake, add 3 ml. of *0.5% iodoxy ammonium benzoate solution* and fill to the mark with *0.167 N hydrochloric acid*. Shake well and let stand exactly 15 minutes. Prepare a reference solution omitting reagent.

Color Reading. Without filtering, read the transmission in a 5-mm. cell.

CALCULATION. Read the vanillin concentration on a curve showing concentrations 0 to 70 γ/ml. as ordinates plotted against the cologarithmic value of per cent of transmission (K value) 0.0 to 0.720 by 0.120 increments and multiply the concentration value by 5.0 to obtain the milligrams of vanillin in 100 ml. of the original extract. Beer's law holds for the above limits.

Phenol Number

Balls and Arana Phospho-Tungstic-Molybdic Colorimetric Method.[81] Process. Dilute 0.5 ml. of the extract to 500 ml.; then to 5 ml. of this add 10 ml. of *0.4 N NaOH* and, with shaking, 3 ml. of *phenol reagent* (p. 754).

Reading. After 5 minutes, read the transmission percentage with respect to water in a Coleman spectrophotometer.

Calculation. From a calibration graph for solutions of vanillin (0 to 0.10 mg.) treated in like manner, obtain the milligrams and percentage of phenol in terms of vanillin; range 3.24 to 9.00%.

Vanilla Resins

Hess Acid Precipitation Method.[82] Process. Dealcoholize 25 to 50 ml. of the sample as directed under Vanillin and Coumarin, Hess and Prescott Method, make up to the original volume, acidify, and allow to stand overnight. Filter on a weighed paper, wash, dry at 100°, and weigh.

In the tentative method of the A.O.A.C., it is specified to add 2 ml. of *1 + 1 hydrochloric acid*, if the solution is already acid, and, if not acid, to add 1 ml. of *hydrochloric acid* in addition to that necessary to give a distinct acid reaction with *litmus paper*. It is also directed to wash the precipitate with *0.05 N hydrochloric acid*, dissolve in warm *ethanol* into a tared dish, evaporate, dry, and weigh.

Tests. The resins from a pure vanilla extract dissolve in *5% sodium hydroxide solution* to a deep red solution, from which on acidifying a precipitate separates. Neither *ferric chloride solution* nor *hydrochloric acid* produces a marked change in color when added to the ethanol solution of the resins. *Lead subacetate solution*, when added to the filtrate from the resins in the quantitative method, produces an exceedingly bulky precipitate and the filtrate from the precipitate is nearly colorless. When tested with *gelatin*, the filtrate from the resins should show a small amount of tannin.

Ethyl Vanillin

Ethyl vanillin differs from vanillin only in that the ethyl group is substituted for the methyl group. If used in imitation flavors in the United States, at least 0.33% must be present and a declaration must appear on the label. Substitution of the less expensive vanillin is regarded as an adulteration.

Chenoweth Peroxide Colorimetric Test.[83] Since the usual methods do not detect ethyl vanillin in pure vanilla extract, Chenoweth (Virginia Dare Extract Co., Brooklyn, N. Y.) proposes a new qualitative test that definitely indicates the addition of as little as 0.5 g. of ethyl vanillin to 3.785 liters of extract.

Process. *Extraction.* Dealcoholize 50 ml. of the sample, treat with *lead acetate solution*, and extract with ether as directed for the Hess and Prescott Gravimetric Method above. Transfer the ether extract to a small beaker, evaporate, and allow to remain overnight in a desiccator.

Precipitation. Add 1 ml. of *2 + 1 hydrochloric acid* and heat in a water bath at 55° until the residue dissolves, then pour the solution into a medium-sized test tube without rinsing, since a quantitative transfer is not required. Add 1 ml. of *3% hydrogen peroxide solution* and shake frequently while the color changes to yellow, brown, and then red. Finally a deep purple color appears and a blue precipitate forms.

Color Formation. After allowing to stand 15 minutes, add 5 ml. of *benzene* and heat in the water bath at 55°. Shake frequently and allow the test tube to remain in the water bath until the lower aqueous layer becomes a dirty yellow-brown (15 to 20 minutes). Remove from the water bath and carefully pour a major portion of the benzene layer into a small dry test tube. If the benzene is

colored violet, ethyl vanillin is present in the original sample; if the benzene is a light or dirty yellow, ethyl vanillin is absent.

Nechamkin Copper-Iron Thiocyanate Test.[84] REAGENTS. *Ethanolic Potassium Hydroxide Solution, 2%,* to which an excess of NH_4SCN has been added.

Copper-Iron Reagent. Mix 1 ml. of 7% $CuSO_4 \cdot 5H_2O$ solution, 4 ml. of 3% ethanolic $FeCl_3 \cdot 6H_2O$ solution, and 5 ml. of ethanol.

PROCESS. Extract the ethyl vanillin-vanillin fraction with *alcohol-free ether.* If not crystalline, purify by the A.O.A.C. method. Dry the residue over sulfuric acid and place 25 mg. in a test tube. Add 3 to 4 ml. of *ethanol,* shake, then add 1 drop each of the *two reagents* and shake gently for 1 minute. Vanillin is indicated by a red color which fades very slightly during several hours and ethyl vanillin alone by a reddish color which fades very rapidly to a light yellow color.

For quantitative determination, compare in a colorimeter with solutions of known vanillin and ethyl vanillin content.

COUMARIN

Leach Iodine Test.[85] Treat the residue after removal of the vanillin, obtained by the Hess and Prescott method, with a few drops of water, warm gently, and add a little *iodine in potassium iodide solution.* If the residue is coumarin, a brown precipitate will form and collect as dark green clots on stirring.

Wichmann Ferric Chloride Test.[86] To 25 ml. of the sample, add 25 ml. of water, acidify with *sulfuric acid* if alkaline, and distil to dryness. Add to the distillate 15 to 20 drops of *1 + 1 potassium hydroxide solution,* evaporate at once to 5 ml., pour into a test tube, then evaporate and fuse in a direct flame. Dissolve the nearly colorless melt, after cooling, in a few milliliters of water, transfer to a small flask, and make slightly acid with *25% sulfuric acid,* keeping the volume within 10 ml. Distil into a test tube containing 4 to 5 drops of *neutral 0.5% ferric chloride solution.* The formation of a purple color, characteristic of salicylic acid, is indicative of coumarin in the extract.

Dean Modification.[87] Erroneous results, due to the presence of salicylic acid in non-alcoholic extracts or saccharin as a sweetener, albeit now seldom if ever used, are obviated by the following details.

Dealcoholize, if necessary, 25 ml. of the sample, add 5 ml. of *1 + 3 ammonium hydroxide,* and separate coumarin by shaking with 15 ml. of *ether* which fails to dissolve salicylic acid, saccharin, and vanillin from an ammoniacal solution. Draw off the aqueous liquid and evaporate the ether layer to dryness on the steam bath. Add 5 drops of *1 + 1 potassium hydroxide solution,* evaporate cautiously to dryness, and fuse the residue at the lowest possible temperature, avoiding blackening, thus converting the coumarin into salicylic acid.

Dissolve the melt in a little water, add *dilute sulfuric acid* to acid reaction, transfer to a test tube, and shake well with 5 ml. of *chloroform.* Pipet the chloroform layer through a plug of cotton into another test tube containing 1 to 2 drops of *ferric chloride solution* diluted with 1 to 2 ml. of water, and note the color after mixing.

NORMAL LEAD NUMBER

The precipitate formed with normal lead acetate consists of lead salts of organic and inorganic acids derived from the vanilla bean. Solutions of vanillin and coumarin, colored in imitation of vanilla, yield practically no precipitate with the reagent.

Winton and Lott Indirect Gravimetric Method.[88] PROCESS. *Lead Determination.* Dilute the 10-ml. aliquot of the filtrate from the lead acetate precipitate, obtained by the Hess and Prescott method for vanillin and coumarin above, with 25 ml. of well-boiled water, add a moderate excess of *sulfuric acid,*

mix with 100 ml. of *ethanol*, and allow to stand overnight. Collect the precipitate of lead sulfate on a Gooch crucible, wash with *ethanol*, dry at a moderate heat, and ignite at low redness for 3 minutes in the oxidizing cone of a Bunsen burner or in an electric muffle.

Blank. To 50 ml. of boiled water in a 100-ml. volumetric flask, add 10 ml. of *standard lead acetate solution*, fill to the mark, mix, pipet 25 ml. into a beaker, and determine the lead as sulfate as in the actual analysis.

CALCULATION. Obtain the normal lead number by the following formula:

$$A = \frac{100 \times 0.6831(S - s)}{5} = 1$$

in which A is the normal lead number, S and s are grams of lead sulfate in the precipitate obtained in the blank and actual analysis respectively, each corresponding to 2.5 ml. of the standard lead acetate solution.

Pure vanilla extract of standard strength (1 g. of bean per 10 ml.) should have a normal lead number of at least 0.40.

Wichmann Method.[89] PROCESS. Measure into a 1-liter round-bottom flask 50 ml. of the sample, 175 ml. of boiled water, and 25 ml. of clear *lead acetate solution* (8 g. per 100 ml.). Place the flask over a hole in an asbestos board that indicates 50 ml. of contents in the flask and distil 200 ml. of liquid into a volumetric flask. Reserve the distillate for ethanol determination and transfer the residue to a 100-ml. volumetric flask by means of *carbon dioxide-free water*, using a rubber-tipped rod for loosening adhering matter. Cool, dilute to volume, mix, and filter through dry paper.

Determine lead as sulfate or chromate in 10 ml. of the filtrate.

Conduct a blank determination, using 5 drops of *glacial acetic acid* in place of the sample and collecting 150 ml. instead of 200 ml. of distillate.

CALCULATION. Correct the weight of the precipitate for that found in the blank and calculate the Wichmann lead number (W) by formula (1) or (2):

(1) $W = PbSO_4 \times 13.66$

(2) $W = PbCrO_4 \times 12.82$

SUGARS

Observe the physical character of the residue obtained in the determination of solids. If there is no evidence of a clammy consistency, due to glycerol, polarize before and after inversion and calculate the sucrose.

If the sucrose content is practically the same as that of the solids, further procedure is usually unnecessary. If, however, there is a marked difference, polarize after inversion at 87° and calculate the approximate glucose content; also determine the reducing sugars and calculate as dextrose. As the vanilla bean is only slightly acid and the extract is prepared without heating, tests for invert sugar are usually superfluous.

If the results of the foregoing tests indicate the presence of glycerol, proceed as with Sweet Wines, Part II, F1.

ETHANOL

Determine as directed for Sweet Wines (Part II, F1).

GLYCEROL

The method for Sweet Wines (Part II, F1) is suitable for vanilla extract. If the extract or flavor contains no sugar, the method for sour wines is satisfactory.

ACIDITY

Titrate 10 ml. of the sample, diluted to 200 ml., with *0.1 N sodium or potassium hydroxide solution*, using *phenolphthalein indicator*. Express the result in milliliters of 0.1 N alkali per 100 ml. of extract.

Colors

Color Value of Extract. Dilute 2 ml. of the sample to 50 ml. with a mixture of equal parts of *ethanol* and water and determine the color value in terms of red (R) and yellow (Y) in a 1-inch cell of the Lovibond Tintometer (Fig. 205). Multiply the reading by 25 and calculate the ratios of the two colors.

Color Value of Lead Filtrate. Determine the color value of the filtrate, obtained by the Hess and Prescott method for vanillin and coumarin (above), directly in a 1-inch cell and multiply the reading by 2 to represent the volume of the original extract. If the solution is very dark, observe in a 0.5- or 0.25-inch cell and multiply by 4 or 8.

Modified Marsh Test. Evaporate 25 ml. of the sample on a steam bath until no considerable amount of alcohol is present, as indicated by the odor. Proceed with the sirup as directed in Part II, F3. Express result as percentage of color insoluble in amyl alcohol.

Tests for Coal-Tar Colors. See Part I, C12.

Ash

Determine *Total Ash*, *Soluble Ash*, *ble Ash*, and *Alkalinity* of each as directed in Part I, C2*f*, using 10 ml. of the sample.

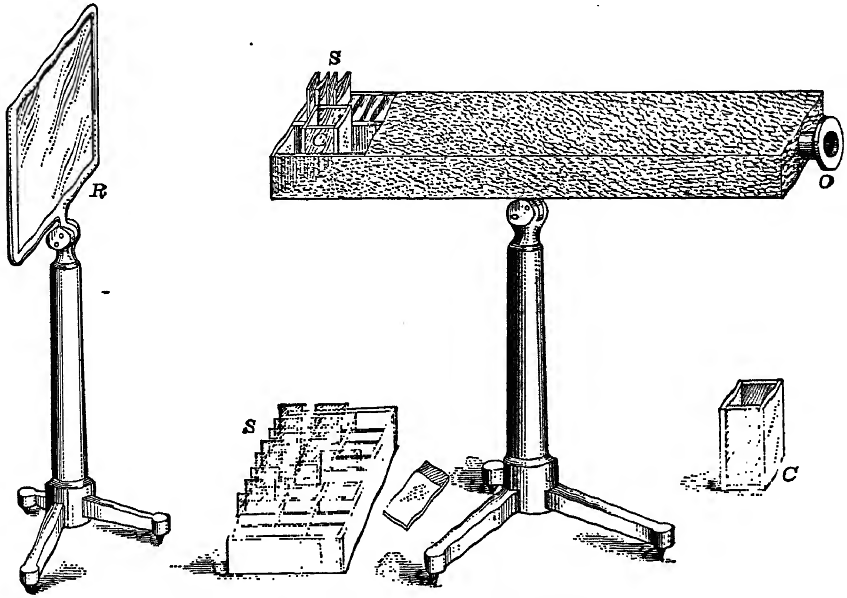


Fig. 205. Lovibond Tintometer.

k. Vanilla Oleoresins

Commercial preparations known as oleoresins are used as the base for the manufacture of extracts and flavors. Wilson and Sale[90] (Bureau of Chemistry, Washington) have analyzed oleoresin made by them from different grades of vanilla beans, also commercial oleoresins. They recommend the following determinations:

(1) On the well-mixed oleoresin, (*a*) ash, (*b*) total alkalinity of ash; (2) on an extract

of the oleoresin in ethanol, (*a*) non-volatile solids at 100°, (*b*) vanillin, (*c*) glycerol; (3) on the water extract of the oleoresin after extraction with ethanol, (*a*) non-volatile solids at 100°, (*b*) caramel; (4) on an extract of the oleoresin in 47.5% ethanol, (*a*) vanilla resin, (*b*) Wichmann lead number.

l. Wintergreen Extract

Although wintergreen oil is defined by the U. S. Standards as being derived from the leaves of the true wintergreen (*Gaultheria procumbens* L.) and wintergreen extract as the 3% by volume solution thereof, neither product is of considerable commercial importance. On the other hand, oil of betula or sweet birch is made in the United States in large quantities by the distillation of the bark of the black birch (*Betula lenta* L.) and constitutes the flavor commonly known as wintergreen. Like true wintergreen oil, it consists almost entirely (up to 99.8%) of methyl salicylate but is optically inactive, whereas wintergreen oil is slightly (up to 1°) levorotatory. Synthetic methyl salicylate is often substituted for both natural products.

Volatile Oil

Howard Centrifugal Method. Follow the directions given by Howard for Lemon Extract above, except that the wintergreen oil, which is heavier than water, is brought into the neck of the Babcock test-bottle by addition of *1 + 2 sulfuric acid* or *saturated salt solution* instead of water.

Methyl Salicylate

Hortvet and West Salicylic Acid Gravimetric Method.[91] Process. *Conversion into Potassium Salicylate.* Pipet 10 ml. of the sample into a 100-ml. beaker and convert the methyl salicylate into potassium salicylate by heating with 10 ml. of *10% potassium hydroxide solution* over a boiling water bath. The completeness of the conversion is indicated by the disappearance of the odor of wintergreen which takes place when the liquid has been reduced to about half the original volume.

Salicylic Acid Liberation. Add to the liquid *10% hydrochloric acid* to acid reaction, cool, and shake in a separatory funnel with 40-, 30-, and 20-ml. portions of *ether*. Filter the combined ether solutions through a dry paper into a tared dish and wash with 10 ml. of *ether*. Evaporate the filtrate and washing at 50°, dry 1 hour in a desiccator, and weigh.

Calculation. Multiply the weight of salicylic acid by 1.101 to convert into methyl salicylate, and by 10 to obtain the weight of the latter in 100 ml. Convert the product into per cent by volume by dividing by 1.18, which is the specific gravity of methyl salicylate.

4. SYNTHETIC FLAVORS

Imitation fruit flavors are various mixtures of ethers (chiefly methyl, ethyl, and amyl radicals combined with aliphatic acids containing from 1 to 10 carbons), aldehydes, ketones, and volatile oils. As a rule the similarity to the true fruit flavors is not close. The isolation of the components in some cases may be simple, involving mere fractional distillation, or exceedingly difficult, demanding the technique of organic research. The nostrils often detect certain constituents such as amyl acetate in banana flavors and methyl anthranilate in grape flavors, the latter being unusually successful imitations.

The investigations of Power and Chesnut, E. K. Nelson, Wilson, and others on the flavoring constituents of natural products and of Wilson on commercial flavors have made possible the isolation and quantitative determination of many of the constituents of fruits and extracts. Details appear in their papers published in the *Journal of the Ameri-*

can Chemical Society and the *Journal of the Association of Official Agricultural Chemists.*

Wilson[92] classifies commercial flavoring constituents, which may be separated by fractioning, as (1) *Non-Volatile Constituents* including (*a*) esters, alcohols, lactones, and other inactive constituents, (*b*) higher fatty acids, benzoic acid, cinnamic acid, (*c*) vanillin, ethyl protocatechuicaldehyde, and phenols, and (2) *Volatile Constituents* including (*d*) sodium salts of free volatile acids, (*e*) semicarbazones of aldehydes and ketones, (*f*) free and combined alcohols, (*g*) lactones and anhydrides, and (*h*) sodium salts of acids combined as esters.

He also gives the method for approximate estimation of (1) *free volatile acids* as acetic, (2) *aldehydes and ketones*, and (3) *volatile esters and lactones* as ethyl acetate.

Ionone

Both α- and β-ionone are isomers of the ketone irone $[C_6H_6(CH_3)_2(CH_3)CH{:}CH \cdot CO \cdot CH_3]$, the chief odorous constituent of orris root and violet.

Wilson Nitrobenzhydrazide Direct Gravimetric Method.[93] This official method (U. S. Food Administration) is applicable to pure solutions of 100 mg. or less in 5 ml. of ethanol.

Process. Add to 5 ml. of the ethanolic solution in a 125-ml. Erlenmeyer flask 95 to 100 mg. of *solid m-nitrobenzhydrazide*, dissolve by cautious warming, dilute with 5 ml. of water, and warm further if cloudy. Add 0.2 ml. of *glacial acetic acid*, stopper lightly, and cool slowly on a wooden surface, noting the formation of crystals in 30 minutes if as much as 20 mg. of β-ionone is present. Let stand 2 to 18 hours, add 5 ml. of water dropwise while rotating the flask, stopper, let stand 1 hour at room temperature, then overnight in the refrigerator. Collect the precipitate on a No. 3 or 4 sintered glass crucible, wash with 30 ml. of *3 + 7 ethanol*, dry at 100°, and weigh.

Calculation. Use the formula: β-ionone-*m*-nitrobenzhydrazide × 0.541 = β-ionone.

Wilson Nitrobenzhydrazide Distillation Modification.[92] The modification is designed for raspberry flavor.

Process. *Distillation.* Steam-distil 205 to 1000 ml. of the sample, containing not more than 100 mg. of β-ionone, into enough water to cover the outlet and collect 500 ml. of liquid.

Extraction. Dilute the distillate in a separatory funnel to about 10% ethanol content. Shake for 2 minutes with sufficient *ether* (150 to 200 ml.) to insure a separation of about 100 ml., draw off the aqueous layer until about 25 ml. remain, then whirl, let settle, and draw off the remainder.

Nitrobenzhydrazide Treatment. Run the ether layer into a 125-ml. Erlenmeyer flask containing 95 to 100 mg. of *m-nitrobenzhydrazide*, let drain 1 minute, and rinse with 10 to 15 ml. of *ether*. Add 0.2 ml. of *glacial acetic acid*, stir, heating if necessary, until the hydrazide is dissolved. After 1 hour, evaporate on a steam bath to about 10 ml. while passing an air current into the flask.

Make in like manner a second and a third extraction of the distillate with 100 ml. each of *ether*, evaporate the extracts to 10 and to 1 to 3 ml. respectively, and add to the main extract.

Separation of β-Ionone-m-Nitrobenzhydrazide. While the flask is still warm, wash down the sides with 5 ml. of *ethanol* from a pipet, and dissolve the residue by heating on a steam bath, avoiding loss by evaporation. Add 5 ml. of water, warm until clear, add 0.2 ml. of *glacial acetic acid*, stopper with a cork, and allow to cool slowly on a wooden surface. After 2 hours, add dropwise 5 ml. of water, mix by rotating, stopper, and keep at room temperature for at least 1 hour, or overnight, then keep in a refrigerator for 12 to 18 hours. Collect the precipitate on a fritted glass crucible of 3 or 4 porosity and

wash with 30 ml. of *3 + 7 ethanol*. Dry in a vacuum oven at 70° and weigh. Use the factor 0.541 for the calculation.

If the precipitate is contaminated with oils, pour 5 ml. of *naphtha* into the crucible in a holder over a test tube in the suction flask, cover, and let stand 5 minutes, then apply suction. Repeat until no more soluble matter is extracted. Transfer to a small beaker and allow the ether to evaporate spontaneously. Examine microscopically all residues and crystals for the properties given below.

Optical Properties of β-Ionone-*m*-Nitrobenzhydrazide. In mass the substance is yellowish, but in ordinary light under the microscope it is essentially colorless and forms crystals in thin rod-like plates often with lath-like or frayed ends, some with six-sided outline. With crossed Nicols, the extinction is parallel and the sign of elongation negative. The refractive indices are the minimum and maximum values $n_\alpha = 1.548$, invariably shown on the elongated fragments when their long dimension is parallel to the vibration plane of the lower Nicol (lengthwise), and $n_\gamma = 1.648$, usually shown on elongated fragments when their long dimension is at right angles to the vibration plane of the lower Nicol (crosswise).

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[60] *J. Am. Chem. Soc.* 1899, **21,** 1132; *U. S. Dept. Agr., Bur. Chem.* 1902, *Bull.* **65,** 73; 1908, *Bull.* **107** (rev.), 159; Official A.O.A.C. Method.

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[69] *Ind. Eng. Chem., Anal. Ed.* 1941, **13,** 166.

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[74] *J. Am. Chem. Soc.* 1899, **21,** 256; Official A.O.A.C. Method.

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[89] *J. Ind. Eng. Chem.* 1921, **13,** 414; *J. Assoc. Official Agr. Chem.* 1925, **8,** 689, 691; 1926, **47,** 87; Official A.O.A.C. Method.

[90] *Ind. Eng. Chem.* 1926, **18,** 283.

[91] *J. Ind. Eng. Chem.* 1901, **1,** 84; Tentative A.O.A.C. Method.

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1. YEAST

Fermentation in bread making is induced by top or compressed yeast which differs structurally (Fig. 148, I) from bottom or beer yeast (Fig. 148, II) and reacts differently in fermentation.

Composition. The following analysis of compressed yeast by Frey [1] shows that it is rich in nitrogenous matter and glycogen, but contains no starch: moisture 73.00, protein 14.15, fat 0.46, glycogen 8.16, cellulose, gum, etc., 1.87, and ash 2.36%, fresh basis. Over half of the dry matter is protein and nearly one-third glycogen.

The principal proteins are *cerevisin* and *zymocasein.* Two purines, *guanine* and *adenine,* and two pyrimidines, *uracil* and *cytosine,* are present.

Proximate Constituents. The usual methods for the determination of *water, protein, fat, fiber,* and *ash* (Part I, C2) are suitable for yeast. Special methods follow.

Starch

Although starch is not a natural constituent of yeast as supplied in bricks to the baker, it is added in small amount to domestic yeast cakes sold in tinfoil wrapping. It may be determined by the Maercker Diastase Method (Part I, C6*a*), after washing with water to remove any water-soluble copper-reducing substances that may be present.

Glycogen

See Part II, H1, H2, and H5.

Gas Production

The amount of carbon dioxide formed under optimum conditions is determined by essentially the same methods as that liberated from a carbonate or baking powder by the action of acid. The methods are classified as (1) *indirect* by calculation from the loss in weight due to fermentation, (2) *gasometric* by measurement of the volume of the gas evolved,[2] (3) *manometric* by measurement of the pressure of the gas evolved,[3] (4) *gravimetric* by weighing the gas evolved after absorption in a potash bulb or soda lime tube, and (5) *volumetric* by back-titration of the excess of alkali in (4).[4]

Only the first of these methods is described herewith, since the others involve the same technique as methods given below under Baking Powder or under Van Slyke, Dillon, Macfadyen, and Hamilton Carboxyl Group Ninhydrin Gasometric Method, Part I, C4*b*.

Meissl Indirect Gas Evolution Gravimetric Method.[5] Apparatus. *Meissl Fermentation Flask* (Fig. 206). A 70- to 80-ml. Erlenmeyer flask equipped with a double-bored stopper carrying (1) an air entrance tube closed with a piece of tubing and a short glass rod and (2) either a small *sulfuric acid bulb* as shown, or a *soda lime tube,* for preventing the escape of moisture.

Reagents. *Yeast Food.* (*A*) Grind and mix thoroughly 25 g. of $(NH_4)_2HPO_4$, 25 g. of K_2HPO_4, and 400 g. of rock candy sucrose. (*B*) Dilute 30 g. of saturated $CaSO_4 \cdot 2H_2O$ solution with 70 g. of water.

Process. Place 4.5 g. of *yeast food A* in the Erlenmeyer flask and dissolve in 50 ml. of *yeast food B.* Thoroughly disperse 1 g. of the sample through the liquid and place the apparatus in a water bath kept at 30°. Shake every 10 minutes for 2 hours, then every 15 minutes for 4 hours. Cool in ice water and aspirate 120 bubbles of air through

the liquid during 1 minute. Dry the outside of the assembly and weigh the whole at room temperature as before.

CALCULATION. Multiply the loss in weight by 100 and divide the product by 1.75, thus obtaining the percentage of fermentation

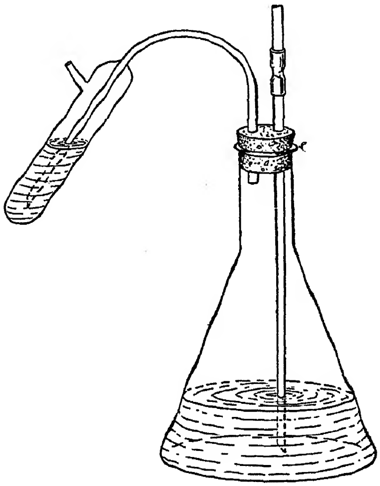


FIG. 206. Meissl Fermentation Flask.

power compared with standard yeast yielding 1 liter of carbon dioxide, which weighs 1.75 g.

NOTE. Some chemists bring the reaction solution to a gentle boil, then cool and aspirate. In any event, comparisons should be made with results on standard material treated exactly in the same manner.

DOUGH EXPANSION

The method is the complement of the dough expansion procedure as applied to flour of unknown expansion, using yeast of known activity. In the present case, the expansion value of the standard flour is known and the yeast activity (gas production value) is unknown. The expansion of the dough furnishes more directly practical information than the mere gas production.

BEER YEAST

Several methods have been described by continental authors for the detection of beer yeast (bottom yeast) from compressed or bread yeast (top yeast).

Küttner and Ulrich Granulation and Odor Tests.[6] Compressed yeast retains its natural odor during storage for 4 days in a closed container, beer yeast, even if only in 10% admixture, however, develops a characteristic sour, stuffy odor. The granules that form in beer yeast in a sludge test persist and differ markedly from the smooth appearance that characterizes compressed yeast on standing.

Bau Raffinose-Copper Reduction Test.[7] Various tests based on the inability of compressed yeast to act on raffinose have been proposed. The Austrian Codex directs to perform the Bau test as follows. Mix 0.4 g. of the yeast in a test tube with 10 ml. of *1% raffinose solution* and allow to stand 24 hours at 30°. Filter, mix 3 ml. of the filtrate with 1 ml. of *Fehling solution*, and heat for 5 minutes in a boiling water bath. If the color of the liquid remains blue, absence of beer yeast is assured.

Herzfeld Raffinose-Gas Formation Test.[8] Mix 10 ml. of *1% raffinose solution* with 1 g. of yeast in an Einhorn fermentation saccharimeter as in urine analysis. After 24 hours of reacting at 30°, the presence of 5% of beer yeast is shown by the production of 4 to 5 ml. of carbon dioxide, whereas compressed yeast never gives over 3 ml. (old process) or 4 ml. (new process).

The test is not infallible and should be confirmed by other tests.

THIAMIN

See Part I, C10.

Riboflavin

Schumacher and Heuser Hydrosulfite Photometric Method.[9] The procedure was developed at the Department of Poultry Husbandry, Cornell University.

Apparatus. The *Photometer* is that devised by Sullivan and Norris (also of Cornell University)[10] for use in the determination of riboflavin in dried milk products.

Reagent. *Sodium Hydrosulfite Reagent.* Dissolve 1 g. of $NaHCO_3$ and 1 g. of $Na_2S_2O_4$ in 20 ml. of cold water.

Process. *Extraction.* Weigh 10 g. of the sample into a 250-ml. Erlenmeyer flask, add 100 ml. of *5% hydrochloric acid*, reflux for 40 minutes, cool, stopper, and store in a refrigerator for 1 hour. Filter the supernatant liquid on a pleated paper, remove an aliquot of 25 ml. to a 50-ml. volumetric flask, add *5 N sodium hydroxide solution* (about 2.25 ml.) to *p*H 3.5 to 4.0, and make up to 49 ml. with water.

Removal of Interfering Colors. Add 1 ml. of *hydrosulfite reagent* as prepared by Hodson and Norris.[11] Transfer to a 250-ml. Erlenmeyer flask, stopper, and after 10 minutes remove the stopper and shake vigorously for 5 minutes, thus reoxidizing the riboflavin to the colored form, then filter with moderate suction through a Gooch crucible.

Color Reading. On 20 ml. of the clear filtrate, make an initial reading (I_1) of the light absorbed, then add 0.6 ml. of *sodium hydrosulfite reagent* and make a second reading (I_2).

Calculation. Subtract I_2 from I_1 and multiply by 1.036, since only 90% of the riboflavin is reduced, then multiply by 20 to obtain the gammas of riboflavin per gram of yeast.

Since the solution follows Beer's law, the following formula for gammas of riboflavin (R) per gram may be used:

$$R = \frac{\left[36.7 \log \frac{I_0}{I_1} - 36.7 \left(\log \frac{I_0}{I_2}\right)(1.0333)\right] 1.036 \times 20}{0.90}$$

in which I_0, I_1, and I_2 are the blank, initial, and reduced readings respectively, 1.0333 corrects for the dilution with 0.6 ml. of sodium hydrosulfite, 1.036 corrects for *p*H of 3.5 to 4.0, 20 is the sample dilution, 0.90 corrects for 90% dilution, and 36.7 is the absorption coefficient as found in standardizing the instrument.

The formula condenses to

$$R = 844.9(I_1 - 1.0333 I_2)$$

in which I_1 and I_2 are log I_0/I_1 and log I_0/I_2 respectively.

Enzymes

According to Kuhn and Münch,[12] the evidence for the existence of the two enzymes, *glucosucrase* and *fructosucrase*, is based on the inhibition resulting from the addition of dextrose or levulose to the medium. Yeast sucrase is inhibited by fructose and the sucrase from *Aspergillus niger* by dextrose. The former hydrolyzes all known gluco derivatives of sucrose with the fructose part intact (gentianose, raffinose, stachyose, and hesperonal), the latter hydrolyzes fructo derivatives of sucrose (melezitose). Many other enzymes are present.

2. BAKING POWDER

Modern baking powder is an immediately acting substitute for yeast. It also differs from yeast in that it liberates carbon dioxide from sodium bicarbonate by the action of chemicals, not by the fermentation of sugar through the agency of unicellular cells.

The liberating chemicals are classed as (1) *acids:* tartaric acid, lactic acid; (2) *acid salts:* potassium hydrogen tartrate (cream of tartar), calcium monophosphate, disodium pyrophosphate; and (3) *neutral salts:* sodium

aluminum sulfate (soda alum or "S.A.S."), calcium lactose.

In addition to sodium bicarbonate and the liberating chemical, the common brands of baking powder contain starch added to prevent deterioration and to standardize the available carbon dioxide.

Formulas. All the formulas which follow are designed to yield 14% of carbon dioxide.

Tartrate Baking Powder. Sodium bicarbonate 27%, cream of tartar 60%, starch 13%. Certain brands on the market consist essentially of a mixture with a small amount of a tartaric acid powder.

Tartaric Acid Baking Powder. Sodium bicarbonate 27%, tartaric acid 24%, starch 49%.

Phosphate Baking Powder. Sodium bicarbonate 27%, monocalcium phosphate 35%, starch 38%.

A pyrophosphate powder requires about 10% more of disodium pyrophosphate ($Na_2H_2P_2O_7$) than of monocalcium phosphate to yield 14% of carbon dioxide.

Alum Baking Powder. Sodium bicarbonate 27%, anhydrous soda alum 27%, starch 46%.

Powders of this type are now seldom found on the market.

Alum-Phosphate Baking Powder. Various mixtures of the last two types.

Reactions. *Tartrate and Tartaric Acid Powders* react as follows:

$$\underset{\text{Potassium bitartrate}}{KHC_4H_4O_6} + \underset{\text{Sodium bicarbonate}}{NaHCO_3} \rightarrow \underset{\text{Potassium-sodium tartrate}}{KNaC_4H_4O_6} + CO_2 + H_2O$$

$$\underset{\text{Tartaric acid}}{} \cdot \underset{\text{Sodium bicarbonate}}{2NaHCO_3} \quad \underset{\text{Sodium tartrate}}{} \quad 2CO_2 + 2H_2O$$

Three theoretical reactions for *monophosphate powders* are given below: (1) proposed by Crampton,[13] (2) and (3) by Patten:[14]

1. $$\underset{\text{Monocalcium phosphate}}{CaH_4(PO_4)_2} + \underset{\text{Sodium bicarbonate}}{2NaHCO_3} \rightarrow \underset{\text{Dicalcium phosphate}}{CaHPO_4} + \underset{\text{Disodium phosphate}}{Na_2HPO_4} + 2CO_2 + 2H_2O$$

2. $$\underset{\text{Monocalcium phosphate}}{3CaH_4(PO_4)_2} + \underset{\text{Sodium bicarbonate}}{4NaHCO_3} \rightarrow \underset{\text{Tricalcium phosphate}}{Ca_3(PO_4)_2} + \underset{\text{Monosodium phosphate}}{} \cdot 4CO_2 +$$

3. $$\underset{\text{Monocalcium phosphate}}{3CaH_4(PO_4)_2} + \underset{\text{Sodium bicarbonate}}{8NaHCO_3} \cdot \quad \underset{\text{Tricalcium phosphate}}{} \quad \underset{\text{Monosodium phosphate}}{} + 8CO_2 + 8H_2O$$

The product of the reaction in a *pyrophosphate powder* is $Na_4P_2O_7 \cdot 10H_2O$.

Alum Powders react as follows:

$$\underset{\text{Sodium aluminum sulfate}}{Na_2Al_2(SO_4)_4} + \underset{\text{Sodium bicarbonate}}{6NaHCO_3} \cdot \quad \underset{\text{Aluminum hydroxide}}{Al_2(OH)_6} \cdot \quad \underset{\text{Sodium sulfate}}{} + 6CO_2$$

Lactate Powder, a product little used in the United States, reacts as follows:

$$\underset{\text{Calcium lactate}}{Ca(C_3H_5O_3)_2} + \underset{\text{Sodium bicarbonate}}{2NaHCO_3} \rightarrow \underset{\text{Sodium lactate}}{2Na(C_3H_5O_3)} + CaCO_3 + CO_2 + H_2O$$

Analytical Methods. From the viewpoint of the baker and housewife only the available carbon dioxide has practical significance. The hygienist lays stress on the

metallic impurities. Although determinations of the other constituents are required only in special investigations, the one seeking experience in the separation of common inorganic elements would look far to find better materials for practice. Tartrate powders yield Rochelle salts, a material often given students for the separation of potash and soda; phosphate powders call for methods used in agricultural laboratories for the determination of phosphoric acid and lime; and alum and alum-phosphate powders offer problems of separation of lime, alumina, and phosphoric acid too often neglected in textbooks of inorganic analysis.

In tartrate and tartaric acid powders, the tartaric acid is determined directly; in tartrate powders it may be calculated from the potash content. The soda (Na_2O) obtained by analysis is equivalent to the sodium bicarbonate originally present in the powder. In the phosphate powder, phosphoric acid (P_2O_5) and lime (CaO) are determined. Formerly calcium sulfate was present as an impurity or was added as a filler, but at present determination of sulfuric acid shows that the phosphate is of remarkable purity. As in the tartrate and tartaric acid powders, the soda (Na_2O) obtained on analysis is equivalent to the sodium bicarbonate in the powder.

Determination of alumina (Al_2O_3) and sulfuric acid (SO_3) should show amounts equivalent to that in soda alum. The soda is partly from the alum and partly from the bicarbonate.

Total Carbon Dioxide

The gravimetric methods depend on the liberation of the gas by acid and its absorption, after drying by calcium chloride, either by caustic alkali in a potash bulb, as in the Knorr assembly [15] or by soda lime in a U-tube, as in the Heidenhain train. The former is specified for the Official Method; [16] the latter was found by Winton, Ogden, and Langley [17] to yield theoretical results on pure Iceland spar and was used in the analysis of representative brands of baking powder.

Heidenhain Soda Lime Gravimetric Method. Apparatus (Fig. 207). *Mulder Apparatus,*[18] as improved by Kolbe, Stobe, and Fresenius and modified by Heidenhain:

A. Soda lime cylinder for removing carbon dioxide from the air drawn through by an aspirator. Above the soda lime is a thick layer of cotton to prevent soda lime dust from being carried over.

B. Cock to regulate rate of aspiration.

C. Capillary contraction.

D. Cylindrical funnel tube narrowed so that the small rubber stopper or tube on *E* forms a tight connection.

E. Tube to regulate the addition of acid by raising or lowering.

F. A 150-ml. evolution flask. (For foaming liquids, use a larger flask.)

G. Condenser tube wound with lead pipe. An all glass condenser is more efficient.

H. Short (18-cm.) and long (50-cm.) U-tubes containing respectively pieces and coarsely ground (No. 18 mesh) calcium chloride. The short tube is refilled when contents become liquid.

K. 18-cm. U-tube containing at *I* an 8-cm. column of pumice stone impregnated with copper sulfate, thoroughly dehydrated at 150°. The remainder of the tube is filled with 18-mesh calcium chloride.

L. Cock to be closed when not in use.

M. Absorption tube about 1.5 cm. in diameter and 12 cm. long, filled with 18 mesh soda lime, except for a little calcium chloride at the left-hand side. It should be refilled when it has gained 0.75 g.

N. Absorption tube, left half filled with 18-mesh soda lime, right half filled with 18-mesh calcium chloride. When the tube has gained 0.1 g. it should be refilled.

O. Guard tube containing calcium chloride (left) and soda lime (right).

P. Indicator tube serving as glycerol trap.

R. Safety bottle to catch water if sucked back from aspirator.

S. Mariette bottle serving as aspirator.

Boil connecting rubber tubes with dilute alkali, wash, dry, lubricate with a trace of castor oil, and wire or tie.

Preparatory for use, pass carbon dioxide through *H* and *K* for several hours and exhaust.

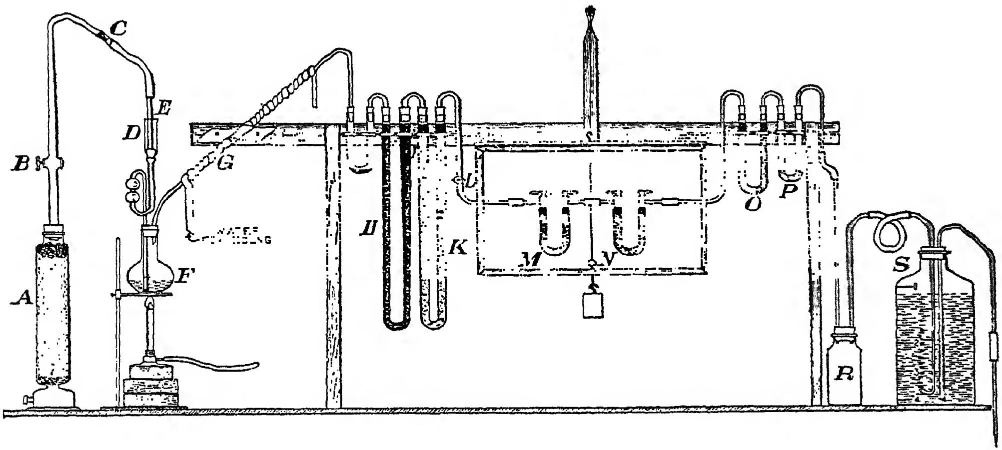


Fig. 207. **Heidenhain Carbon Dioxide Apparatus.**

Reagents. *Calcium Chloride*. Dehydrate at 200° lumps of the grade designed for ultimate organic analysis. Grind and sift to pass a No. 18 mesh, rejecting the portion that passes a No. 30 mesh. Fill tubes from the same lot so that the air entering and leaving will be uniform in moisture content.

Soda Lime. Prepare as recommended by Benedict and Tower [19] as follows. Heat in an iron kettle 1 kg. of commercial sodium hydroxide with 500 to 600 ml. of water. Add to the thin paste while hot 1 kg. of powdered quicklime, stirring with an iron rod to break up lumps and aid in drying. After cooling, grind and sift as directed above for calcium chloride. Avoid over drying.

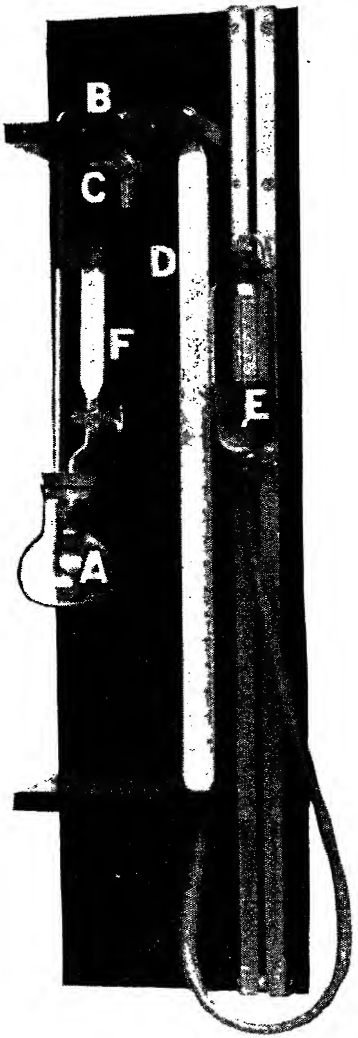
Process. *Gas Generation*. Allow tubes *M* and *N* to acquire room temperature, open the cocks for a moment to equalize pressure, weigh, and connect with the apparatus. Test the connections by closing *A* at the bottom, opening all cocks and aspirating until the liquid in *P* ceases to move which should be in a few minutes. Disconnect the aspirator and close *B*, remove *F* and weigh into it 2 g. of baking powder or about half that quantity of calcite or sodium bicarbonate. Connect with the train and run water through the condenser. Add through *D*, by lifting the tube, in small portions, an excess of *1 + 1 hydrochloric acid* and a suitable amount of well-boiled water.

Aspiration. Heat cautiously to boiling and boil gently. When no more air passes through *P*, begin to aspirate, continuing the aspiration until the water stops running. Open *B* cautiously and adjust the rate of flow by raising or lowering the siphon in *S* to

half the safe speed. After M feels cool to the touch, increase the current to full safe speed and aspirate to a total of 3 liters, with continual boiling. Cool the tubes to room temperature, open for a moment, and weigh.

Establish the safe speed by carrying out a blank analysis, omitting the substance. Aspirate at the rate of 50 ml. per minute with 2 liters of air and weigh the tubes. If together they have lost weight, reduce the speed. What tube M loses, tube N should gain.

Dietrich Gasometric Method.[20] Although not so exact as the gravimetric method, the method which depends on measurement of the gas is rapid and adequate for many pur-



Courtesy of Methods of Analysis, A.O.A.C., 1935, p. 185

FIG. 208. Chittick Gasometric Carbon Dioxide Apparatus.

APPARATUS. In its simplest form the assembly consists of (1) a *salt-mouth bottle*, serving as an evolution flask, connected through one hole of a triple-bored rubber stopper by glass and rubber tubes with (2) a 50- or 100-ml. *Mohr buret* (without stopcock) inverted in a cylinder of mercury, serving as a gasometer. Through one of the two other holes passes a short piece of glass tubing connected with a short piece of rubber tubing closed by a pinchcock; through the third hole passes a thermometer. A thick-walled test tube, cut to a suitable length and introduced into the salt-mouth bottle in an upright position, serves as an acid container.

PROCESS. Weigh into the salt-mouth bottle a suitable amount of the sample and measure into the tube 5 ml. of *25% hydrochloric acid*. Open the stopcock, adjust the level of the mercury within the buret to correspond with that without, then close the stopcock and immediately tip the bottle so that the acid empties onto the sample. Allow about 10 minutes for the evolution of the gas and adjustment of the temperature, then adjust the level of the mercury within the buret as before and take the reading, also that of the temperature and barometric pressure.

CALCULATION. Dietrich's calculation tables are given by Fresenius, but Parr's table herewith answers the purpose, provided the displacement liquid as prepared for the Chittick method is substituted for mercury.

Density of Carbon Dioxide (Parr) *

(Weight in milligrams of 1 ml. of carbon dioxide corrected for aqueous vapor and barometer readings on glass scale)

mm.	20°	21°	22°	23°	24°	25°	26°	27°	28°	29°	30°
700	1.6462	1.6370	1.6278	1.6195	1.6112	1.6021	1.5930	1.5837	1.5744	1.5649	1.5554
702	.6510	.6419	.6327	.6243	.6160	.6068	.5977	.5884	.5791	.5696	.5600
704	.6558	.6467	.6376	.6292	.6207	.6116	.6025	.5931	.5838	.5742	.5647
706	.6607	.6516	.6425	.6340	.6254	.6163	.6072	.5979	.5885	.5789	.5693
708	.6655	.6564	.6474	.6388	.6302	.6211	.6119	.6026	.5932	.5836	.5740
710	.6703	.6613	.6522	.6436	.6350	.6258	.6166	.6073	.5978	.5882	.5786
712	.6751	.6662	.6571	.6485	.6397	.6305	.6214	.6120	.6025	.5929	.5832
714	.6799	.6710	.6620	.6533	.6444	.6353	.6261	.6167	.6072	.5976	.5879
716	.6848	.6759	.6670	.6581	.6492	.6400	.6308	.6215	.6119	.6023	.5925
718	.6896	.6807	.6718	.6629	.6540	.6448	.6356	.6262	.6166	.6069	.5972
720	.6944	.6856	.6767	.6678	.6587	.6495	.6403	.6309	.6213	.6116	.6018
722	.6992	.6904	.6815	.6726	.6635	.6543	.6450	.6356	.6260	.6163	.6065
724	.7041	.6953	.6863	.6773	.6682	.6590	.6497	.6403	.6307	.6210	.6111
726	.7089	.7001	.6911	.6821	.6730	.6638	.6544	.6450	.6354	.6256	.6157
728	.7137	.7049	.6959	.6869	.6778	.6685	.6591	.6497	.6401	.6303	.6204
730	.7185	.7097	.7007	.6917	.6825	.6732	.6638	.6544	.6448	.6350	.6251
732	.7233	.7145	.7055	.6964	.6872	.6779	.6685	.6591	.6494	.6396	.6297
734	.7282	.7193	.7103	.7012	.6920	.6827	.6733	.6638	.6541	.6443	.6343
736	.7330	.7241	.7151	.7060	.6968	.6875	.6780	.6685	.6588	.6490	.6390
738	.7378	.7289	.7199	.7107	.7015	.6922	.6827	.6732	.6635	.6537	.6437
740	.7426	.7337	.7247	.7155	.7063	.6969	.6874	.6778	.6681	.6583	.6483
742	.7475	.7385	.7295	.7203	.7111	.7017	.6922	.6826	.6729	.6630	.6530
744	.7523	.7433	.7342	.7250	.7158	.7064	.6969	.6873	.6776	.6677	.6577
746	.7571	.7481	.7390	.7298	.7206	.7112	.7016	.6920	.6822	.6723	.6623
748	.7619	.7529	.7438	.7346	.7253	.7159	.7063	.6967	.6869	.6770	.6670
750	.7667	.7577	.7486	.7394	.7301	.7206	.7110	.7014	.6916	.6817	.6716
752	.7716	.7625	.7534	.7441	.7348	.7254	.7158	.7061	.6963	.6864	.6763
754	.7764	.7673	.7582	.7489	.7396	.7301	.7205	.7108	.7010	.6910	.6809
756	.7812	.7721	.7630	.7537	.7443	.7348	.7252	.7155	.7057	.6957	.6856
758	.7861	.7770	.7678	.7585	.7491	.7396	.7300	.7202	.7104	.7004	.6903
760	.7909	.7818	.7725	.7632	.7538	.7443	.7347	.7249	.7150	.7050	.6949
762	.7957	.7866	.7773	.7680	.7586	.7490	.7394	.7296	.7197	.7097	.6996
764	.8005	.7914	.7821	.7728	.7633	.7538	.7441	.7343	.7244	.7144	.7042
766	.8053	.7962	.7869	.7776	.7681	.7585	.7488	.7390	.7291	.7191	.7089
768	.8102	.8010	.7917	.7823	.7728	.7633	.7535	.7437	.7338	.7237	.7135
770	.8150	.8058	.7965	.7871	.7776	.7680	.7582	.7484	.7385	.7284	.7182

* *J. Am. Chem. Soc.* 1909, **31**, 237. Calculated from 1.976 = weight of 1 liter of CO_2 at 0° C., 760 mm. pressure, and 41° latitude. In *Methods of Analysis, A.O.A.C.*, values for 10° to 19° are also given.

Chittick Gasometric Method.[21] Apparatus (Fig. 208). The *evolution flask* (*A*) is of Pyrex glass and has a capacity of 250 ml. The *acid buret* (*F*) is graduated at 5-ml. intervals to 25 ml. A glass tube passes through one hole of the stopper of the evolution flask and is connected with the glass tube at *B* by a rubber tube, thus permitting shaking. The gasometric tube (*D*) has the zero point 25 ml. below the top, allowing for graduation 0 to

FACTORS FOR GASOMETRIC DETERMINATION OF CARBON DIOXIDE (CHITTICK) *

(Based on sample weighing 1.7000 g.)

°C	20.0°	21.0°	22.0°	23.0°	24.0°	25.0°	26.0°	27.0°	28.0°	29.0°	30.0°
mm.											
700	0.96835	0.96294	0.95753	0.95265	0.94776	0.94241	0.93706	0.93159	0.92612	0 92053	0.91494
702	97118	96582	.96041	95547	.95059	94518	.93982	.93435	92888	.92329	.91765
704	97400	.96865	.96329	95835	.95335	94800	.94265	.93712	.93165	.92600	.92041
706	.97688	.97153	.96624	.96118	.95612	95076	94541	.93994	.93441	.92876	.92312
708	.97971	.97435	96912	96400	95894	95359	.94818	.94271	.93718	.93153	.92588
710	.98253	97724	97195	.96682	.96176	.95635	.95094	.94547	93988	.93424	.92859
712	.98535	.98012	.97483	.96971	.96453	.95912	95376	94824	.94265	.93700	.93129
714	.98818	.98294	.97771	97253	.96729	.96194	.95653	95100	.94541	.93976	.93406
716	.99106	.98582	.98065	.97535	.97012	96471	95929	.95382	.94818	.94253	.93676
718	.99388	.98865	.98348	.97818	.97294	.96753	.96212	.95659	.95094	.94524	.93953
720	.99671	.99153	.98636	.98106	.97571	.97029	.96488	.95939	.95371	.94800	.94224
722	.99953	.99435	.98918	.98388	97853	.97312	.96765	.96212	.95647	.95076	.94500
724	1.00241	.99724	.99200	.98665	.98129	.97588	.97041	.96488	.95924	.95353	.94771
726	.00524	1.00006	.99483	.98947	.98412	.97871	.97318	.96765	.96200	.95624	.95041
728	.00806	.00288	.99765	.99229	.98694	.98147	.97594	.97041	.96476	.95900	.95318
730	.01088	.00571	1.00041	.99512	.98971	.98424	97871	.97318	96753	.96176	.95594
732	.01371	00853	.00324	.99788	.99247	98700	.98147	.97594	.97024	.96447	.95865
734	.01659	.01135	.00606	1.00071	.99529	.98982	.98429	.97871	.97300	.96724	96135
736	.01941	.01418	.00888	.00353	.99812	.99265	.98706	.98147	.97576	.97000	.96412
738	.02224	.01700	.01171	.00629	1 00088	.99541	.98982	.98424	.97853	.97276	.96688
740	.02506	.01982	.01453	.00912	00371	99818	.99259	.98694	.98124	.97547	.96959
742	.02794	.02265	.01735	.01194	.00653	1.00100	.99541	.98976	.98406	.97824	.97235
744	.03076	.02547	.02212	.01471	.00929	.00376	.99818	.99253	.98682	.98100	.97512
746	.03359	.02829	.02294	.01753	.01212	.00659	1.00094	.99529	.98953	.98371	.97782
748	.03641	.03112	.02576	.02035	.01488	.00935	.00371	.99806	.99229	.98647	.98059
750	.03924	.03394	.02859	.02318	.01771	.01212	.00659	1.00082	.99506	.98924	.98329
752	.04212	.03676	03141	.02594	.02047	.01494	.00929	.00359	.99782	.99200	.98606
754	.04494	.03959	.03424	.02876	02329	.01771	.01206	.00635	1.00059	.99471	.98876
756	.04776	.04241	.03706	.03159	.02606	.02047	.01482	.00912	.00335	.99747	.99153
758	.05065	.04529	.03988	.03441	.02888	.02329	.01765	.01188	.00612	1.00024	.99429
760	.05347	.04812	.04265	.03718	.03165	.02606	.02041	.01465	.00882	.00294	.99700
762	.05629	.05094	.04547	.04000	.03447	.02882	.02318	.01741	.01159	.00571	.99976
764	.05912	.05376	.04829	.04282	.03723	.03165	.02594	.02018	.01435	.00847	1.00247
766	.06194	.05659	.05112	.04565	.04005	.03441	.02871	.02294	.01712	.01124	.00524
768	.06482	.05941	.05394	.04841	.04282	.03724	.03147	.02571	.01988	.01394	.00794
770	.06765	.06224	.05676	.05123	.04564	.04000	.03424	.02847	.02265	.01671	.01071

* Calculated from 1.976 = weight of 1 liter CO_2 at 0° C., 760 mm. pressure, and 41° latitude by Parr's formula (*J. Am. Chem. Soc.* 1909, **31**, 237). In *Methods of Analysis, A.O.A.C.*, corrections from 15.0° to 35.0° by 0.5° are given.

25 ml. upward and 0 to 200 ml. downward. The leveling bulb (*E*) has a capacity of 300 ml.

REAGENT. *Displacement Solution.* To a solution of 100 g. of NaCl or $Na_2SO_4 \cdot 10H_2O$ in 350 ml. of water, add about 1 g. of $NaHCO_3$ and 2 ml. of methyl orange, then $1 + 5$ H_2SO_4 to a decidedly pink color and stir until all CO_2 is removed. The solution seldom needs replacing.

PROCESS. Weigh 1.7 g. of the sample into the evolution flask, connect with the double-bored stopper, open the stopcock and by means of the leveling bulb (containing the displacement solution) adjust the level of the liquid in the gasometer (*D*) to 10 ml. above 0. Allow 1 to 2 minutes for equalizing the temperature and barometric pressure within, then close the stopcock and lower the leveling bulb to reduce the internal pressure. Slowly run into the evolution flask from the buret (*F*) 10 ml. of *1 + 5 sulfuric acid*, always keeping the leveling solution in the bulb below that in the gasometer. To secure complete evolution, rotate, then vigorously agitate, the evolution flask. After allowing to stand 5 minutes, adjust the liquid in the gasometer so as to be on a level with that in the bulb, then read the volume of the gas, also the temperature and barometric pressure.

CALCULATION. Multiply the number of milliliters of gas by the factor given in the Chittick table herewith for the observed temperature and barometric pressure. Divide the corrected reading by 10 to obtain the percentage by weight of carbon dioxide in the sample.

Residual Carbon Dioxide

McGill-Catlin Gravimetric Method;[22] **Winton, Ogden, and Langley Modification.**[23] PROCESS. Weigh 2 g. of baking powder into a flask suitable for the subsequent determination of carbon dioxide, add 20 ml. of cold water, and allow to stand 20 minutes. Heat in a boiling water oven with occasional shaking for 20 minutes. Drive off the last traces of gas from the semi-solid mass by heating quickly to boiling and boil for 1 minute. Aspirate until the air in the flask is thoroughly changed and determine the residual carbon dioxide by absorption as directed for the Soda Lime Gravimetric Method above.

Available Carbon Dioxide

Subtract the percentage of residual carbon dioxide from that of the total carbon dioxide, both obtained by the same gravimetric method.

NOTE. Advance notice of Order S.R.O. No. 46, effective in England after March 16, 1944, establishes 8% as the minimum for available carbon dioxide and 1.5% as the maximum for residual carbon dioxide. The method specified for residual carbon dioxide is as follows:

Treat 2 g. of baking powder or golden raising powder with 25 ml. of water and evaporate to dryness on a boiling water bath. Repeat the addition of 25 ml. of water and evaporation to dryness in like manner. Determine the carbon dioxide evolved from the residue by addition of dilute sulfuric acid at room temperature and completing the evolution by boiling or by means of reduced pressure.

Ammonia

See Part I, C4*f*. Since organic nitrogen is absent, a few milliliters of *sodium hydroxide solution* may be used in place of magnesium oxide.

Tartaric Acid

Wolff Resorcin Test (Tentative).[24, 25] Remove starch, if present, by shaking 2 g. of the sample for 1 to 2 minutes with 100 ml. of cold water, allowing to settle, and decanting off the supernatant liquid onto a filter. Evaporate the filtrate to dryness, mix the residue with a few crystals of *resorcin*, add 1 ml. of *sulfuric acid*, and heat cautiously. A rose-red color, discharged on dilution with water, appears if tartaric acid, free or combined, is present in the sample.

Silver Nitrate Test for Free Tartaric Acid (Official).[25] Extract 5 g. of the sample with *absolute ethanol* and evaporate the extract. Dissolve the residue in *1 + 10 ammonium*

hydroxide, transfer to a test tube, add a large crystal of *silver nitrate*, and heat gently. The presence of free tartaric acid is indicated by the formation of a silver mirror. The absolute ethanolic extract may be tested also with *resorcin* as directed above.

Total Tartaric Acid

Goldenberg-Geromont-Heidenhain Ethanol Precipitation Volumetric Method.[26] This method is well suited for the determination of total tartaric acid in tartrate and tartaric acid powders, but not for phosphate or alum phosphate powders or powders containing calcium in any form.

Process. *Acetic Acid Treatment.* Place 2 g. of the sample in a shallow porcelain dish, add *potassium carbonate* sufficient to combine with all tartaric acid not combined as potassium bitartrate, and mix with 15 ml. of water. Run into the mixture from a graduated tube or buret *liquified glacial acetic acid* until effervescence ceases, then an additional amount double that first added, and stir for half a minute with a glass rod bent at the end to facilitate agitation.

Ethanol Precipitation. Add 100 ml. of *ethanol* and stir violently for 5 minutes and after allowing to settle at least 30 minutes collect the precipitate on a Gooch crucible provided with a thin mat of filter paper pulp. Wash with *ethanol* until 2 ml. of the filtrate do not react with *litmus tincture* diluted with water.

Titration. Transfer the precipitate and paper to a casserole and dissolve in 50 ml. of hot water. Partially neutralize with *standard 0.2 N potassium hydroxide solution*, leaving the solution distinctly acid, boil for 1 minute, and cool. Finish the titration with *0.2 N alkali*, using *phenolphthalein indicator*, adding 0.2 ml. as a correction.

Calculation. Use the formula: 1 ml. of $0.2KOH$ = 0.026406 g. of $C_4H_4O_5$ (tartaric anhydride) = 0.03001 g. of $H_2C_4H_4O_6$ (tartaric acid) = 0.03763 g. of $KHC_4H_4O_6$ (potassium bitartrate).

Standardize the potassium hydroxide solution against pure dry potassium bitartrate.

Examples. The following percentages of potassium bitartrate were reported by Winton, Ogden, and Langley,[27] as calculated respectively from the total tartaric acid by this method and from the potassium oxide: I, 69.31 and 69.37; II, 54.62 and 54.29; III, 60.19 and 60.20; IV, 53.10 and 53.17; and V, 50.15 and 50.58.

Total, Free, and Combined Tartaric Acid

Hartmann Dual Ethanol Precipitation Volumetric Method.[28] The Goldenberg-Geromont-Heidenhain method, slightly modified, was long an official method, but in the 1940 revision it was displaced by the Hartmann method, which employs two alkalies.

Process. *Solution.* To 2.5 g. of the sample in a 250-ml. volumetric flask, add 100 ml. of water at about 50°. Allow to stand at room temperature for about 30 minutes with occasional shaking, then cool, dilute to the mark, shake vigorously, and filter through a pleated paper. Pipet two 100-ml. aliquots of the clear liquid into 250-ml. beakers and evaporate to about 20 ml.

A. Potassium Tartrate Precipitation and Titration. To one portion, add 3.5 ml. of *1.0 N potassium hydroxide solution*, mix well, then add 2 ml. of *glacial acetic acid.* Again mix and add 100 ml. of *ethanol* with constant stirring. Cool to about 15°, stirring for about 1 minute, and store in the refrigerator overnight. Collect the precipitate in a Gooch crucible on a thin well-tamped mat of asbestos, rinsing the beaker with 75 ml. of ice-cold *80% ethanol.* Finally wash down the sides of the crucible with 25 ml. of *ethanol* and suck dry. Transfer the contents of the crucible to the original beaker, using 100 ml. of hot water. Titrate with *standard 0.1 N alkali*, using *phenolphthalein indicator*. Designate the

number of milliliters of standard alkali used as A.

B. Sodium Tartrate Precipitation and Titration. Proceed as under A, but substitute *1.0 N sodium hydroxide solution* for 1 N potassium hydroxide solution. Designate the number of milliliters of standard alkali used

CALCULATION. Obtain the percentages of total tartaric acid (T), cream of tartar (C), and free tartaric acid (F) respectively by the following formulas:

$$T = 1.5(A + 0.6)$$

$$C = 1.88(B + 0.6)$$

$$F = 1.5(A - B)$$

in which 0.6 is the number of milliliters of 0.1 N alkali corresponding to the solubility of the cream of tartar in the solution.

Anderson, Rouse, and Letonoff Ferrous Sulfate-Peroxide Colorimetric Method.[29] As first noted by Fenton,[30] when a tartrate is treated with ferrous sulfate, hydrogen peroxide, and sodium hydroxide a violet color is formed which appears to be specific, at least citric, succinic, malic, and oxalic acids do not give the test. The method is applicable in the presence of aluminum salts, but not of calcium or phosphate. The following details were elaborated at the Pennsylvania State College.

APPARATUS. *Colorimeter.*

REAGENTS. *Ferrous Sulfate Solution,* 1%. Dissolve 1 g. of $FeSO_4 \cdot 7H_2O$ in 80 ml. of water, heating gently and stirring. Cool, transfer to a 100-ml. volumetric flask, and make up to volume.

Standard d-Tartaric Acid Solution, exactly 16%. To 5 ml. of a standard stock 16% *d*-tartaric acid in a 100-ml. volumetric flask, add 10.66 ml. of 1.0 N NaOH solution and make up to volume; 100 ml. = 0.80 g. of tartaric acid, pH 6.2.

PROCESS. *Solution.* Weigh into a small beaker 2 g. of the sample, add water dropwise, until carbon dioxide ceases to be evolved, then add 45 ml. of water and stir thoroughly to dissolve the tartrates present. Filter into a 100-ml. volumetric flask, wash the residue with three portions of 15 ml. each of water, and make up to volume with water. Determine the pH of the solution colorimetrically, using *chlorophenol red indicator.* If the pH varies from 6.2 by more than ± 0.5, prepare another sample and adjust the pH to 6.2 before making up to volume. As a rule tartrate powders need no adjustment.

Color Formation. Pipet 10 ml. of the solution into a 25-ml. volumetric flask, add 0.2 ml. of *1% ferrous sulfate solution* and 0.2 ml. of *3% hydrogen peroxide solution,* and mix thoroughly. After the color, which on the addition of hydrogen peroxide is yellow, becomes brown, place the flask in an ice bath until the brown color disappears and a definite lavender appears. Add immediately 5 ml. of *1.0 N sodium hydroxide solution,* stopper, mix by inversion twice, and cool in the ice bath for 10 minutes.

Color Comparison. Mix the contents of the flask by inversion twice and compare in a colorimeter with a standard solution, prepared simultaneously, containing 10 ml. of the *standard d-tartaric acid solution.*

CALCULATION. Obtain the per cent of tartaric acid (P) by the formula

$$P = \frac{S \times 0.08 \times 10 \times 100}{U \times W}$$

in which S and U are the readings of the standard and unknown respectively, and W is the weight of the sample.

EXAMPLES. The average results reported on 2 samples of tartrate baking powder by the colorimetric and the *A.O.A.C. Methods* are (1) 39.76 and 39.56 and (2) 34.56 and 34.24% respectively. The lower results by the second method are attributed to the incomplete precipitation of potassium acid tartrate or its slight solubility in the ethanol.

Free Tartaric Acid

Hartmann Direct Determination Method.[31] Reagent. *Saturated Ethanol.* Shake vigorously for several minutes 50 g. of finely powdered purest $KHC_4H_4O_6$ with 100 ml. of ethanol and 100 ml. of water. Let stand 15 minutes with occasional shaking and collect the salt in a Büchner funnel. Wash with 200 ml. of 1 + 1 ethanol, then with full strength ethanol, and finally with ether. Dry in a boiling water oven. Treat 5 g. with 500 ml. of absolute ethanol for 2 hours with occasional shaking. Filtering is unnecessary. A blank, using 50 ml. of chloroform and 150 ml. of the saturated ethanol, should not require more than 0.15 ml. of 1 *N* alkali to neutralize 100 ml. of the mixture.

Process. *Chloroform Digestion.* Weigh 1.25 g. of the sample into an absolutely dry 200-ml. volumetric flask, add 50 ml. of *chloroform,* and let stand 5 minutes with occasional shaking. Add 100 ml. of the *saturated ethanol,* shake for 5 minutes, and let stand 30 minutes with frequent shaking. Dilute to the mark with *saturated ethanol,* shake a few minutes, and filter through a pleated paper.

Titration. Pipet 100 ml. of the clear filtrate into a beaker and titrate with *0.1 N alkali,* using *phenolphthalein indicator.*

Calculation. Multiply the number of milliliters of alkali required by 1.2 to obtain the percentage of free tartaric acid.

Starch

Acid Hydrolysis Copper Reduction Method (Official). If the powder contains lime, either as an acid phosphate (phosphate and alum-phosphate powders) or as sulfate, it should be removed previous to copper reduction as follows. Digest 5 g. of the sample in a 500-ml. volumetric flask with 200 ml. of *3% hydrochloric acid* for 1 hour at room temperature with frequent shaking. Allow to settle, filter on a hardened 11-cm. paper that completely retains the starch, rinse the flask once, and wash the paper at least twice with cold water. Wash back into the flask any starch that may have passed over into the paper by means of 200 ml. of water delivered from the jet of a small wash bottle, add 20 ml. of *25% hydrochloric acid* (sp.gr. 1.125), and treat as directed for the Sachsse Method (Part I, C6*a*).

If no lime is present (tartrate and tartaric acid powders), treat the 5 g. of the sample directly with 200 ml. of water and 20 ml. of *25% hydrochloric acid.*

McGill Cold Acid Direct Gravimetric Method.[32] This method, slightly modified, was found by Winton, Ogden, and Langley [33] to yield results on tartrate and tartaric acid powders closely agreeing with those by the copper reduction method. On phosphate, alum, and alum-phosphate powders, the results usually were satisfactory, but on some powders were over 2% too high.

Process. Treat 1 g. of the sample with 150 ml. of *3% hydrochloric acid* at room temperature for 24 hours with frequent shaking or, if a mechanical shaking device is at hand, shake continuously. Collect the insoluble residue on a Gooch crucible (which need not be tared), wash thoroughly with cold water, then once with *ethanol* and once with *ether.* Heat at 110° for 4 hours, which usually is sufficient for constant weight, then burn to whiteness and weigh again.

The difference in the two weights represents the starch.

Sand

Determination of Sand and Preparation of Solution for Ash Analysis. Reduce 5 g. of the sample in a platinum or silver dish to charcoal at a heat below redness. Exhaust the carbonaceous mass by boiling with *dilute hydrochloric acid,* filtering through an ashless paper into a 500-ml. volumetric flask, and washing with hot water. Return the residue on the paper to the dish and complete the

incineration at a red heat until the ash is white. Exhaust as before and add the filtrate to that previously obtained.

Ignite the paper and the washed insoluble residue and weigh.

Calculate the percentage of acid-insoluble matter (sand).

Dilute the filtrate from the sand to 500 ml. and reserve for the determination of aluminum, calcium, potassium, sodium, sulfur, and phosphorus in aliquot portions.

See also, in Part I, C8*a*, the scheme for ash analysis on which several of the following quantitative methods for the determination of mineral constituents are based.

Leach Sodium Aluminate Test.[34] This test is based on the formation of sodium aluminate ($Na_2Al_2O_4$), which is soluble in water, on ignition of soda alum with sodium bicarbonate and the precipitation of aluminum hydroxide on addition of ammonium chloride to the solution, free ammonia and common salt being other products of the reaction. The sodium phosphate formed by the ignition of a phosphate powder also is soluble, but no phosphate is precipitated except in the presence of aluminum salts. Calcium di- and triphosphates are insoluble in water.

PROCESS. Ignite to partial or complete fusion 2 g. of the sample, extract with hot water, filter, and add to the filtrate *ammonium chloride*, noting if a precipitate forms when the solution has a marked odor of ammonia.

In order to meet the possibility of an unusual combination, the test should be verified by applying the reaction for the quantitative method and the Hammett and Sottery Test as given below.

Hammett and Sottery Aurin Tricarboxylic Acid Test.[35] Application is made (Columbia University) of the formation in the presence of an acetic acid-acetate buffer of a bright red aluminum lake of aurin tricarboxylic acid which, unlike the corresponding chromium lake, retains its color for a time after ammonium hydroxide is added to alkaline reaction.

REAGENT. *Aurin Tricarboxylic Acid Reagent.* Prepare a 0.1% solution of the ammonium salt of the acid.

PROCESS. Dissolve in 5 ml. of *1.0 N hydrochloric acid* the precipitate obtained in the Leach test or that formed in a cold hydrochloric acid solution by ammonia. Add 5 ml. of *3 N ammonium acetate solution* and 5 ml. of *aurin tricarboxylic acid reagent.* Mix and allow time for the formation of the lake, then make alkaline with *ammonium hydroxide* containing *ammonium carbonate.* The persistence of the red precipitate indicates the presence of aluminum.

Quantitative Method. The method is essentially the same as described for ash analysis (Part I, C8*b*), but the precipitate of mixed aluminum and iron phosphate obtained in the analysis of alum and alum-phosphate powders consists chiefly of aluminum phosphate with usually so little iron phosphate that a close approximation to the truth may be reached by applying the factor for Al_2O_3. In high grade phosphate powders, the precipitate is usually small and consists, as in ash analysis, essentially of iron phosphate.

Hess and Campbell Phenylhydrazine Gravimetric Method.[36] This method (University of Michigan) differs from that given below for aluminum and iron chiefly in that phenylhydrazine is used to precipitate the alumina as phosphate.

PROCESS. *Solution.* Incinerate 3 g. of the sample and prepare a solution as directed under Sand above, except that the residue, after dissolving the ash with acid, is ignited with 2 g. of *sodium carbonate* and a solution of the fused mass in acid is added to the main solution.

Phenylhydrazine Precipitation. To a 100-ml. aliquot, representing 1 g. of the baking

powder, add *1 + 10 ammonium hydroxide* until a slight precipitate forms, then clear with a few drops of *dilute hydrochloric acid.* Add to the solution about 10 drops of *saturated ammonium bisulfite solution* (prepared by passing SO_2 gas into 1 + 1 ammonium hydroxide) and heat. Add 1 or 2 ml. of *phenylhydrazine* until a permanent precipitate forms, also add *1 + 10 ammonium hydroxide* if necessary to form the precipitate, followed by a few more drops of *phenylhydrazine.* Let settle, filter while warm, and wash with *wash water* containing 5 to 10% of *phenylhydrazine bisulfite solution* prepared by adding saturated SO_2 water to phenylhydrazine until the crystals that form nearly dissolve. Ignite the precipitate, consisting of a mixture of aluminum oxide and aluminum phosphate, and weigh.

Determination of Phosphoric Acid. Employ the Molybdic Gravimetric Method, Part I, C8*a*.

Calculation. Obtain the weight of aluminum by subtracting the weight of phosphoric acid from that of the weight of the phenylhydrazine precipitate.

Aluminum and Iron

(Phosphate, Alum, and Alum-Phosphate Powders)

Phosphate Gravimetric Method (Official). Process. *Precipitation as Phosphates.* To an aliquot of 100 ml. of the solution, prepared as described above under Sand, representing 1 g. of the sample, add *sodium phosphate solution* in excess of that required to form aluminum phosphate with the alumina present, then *ammonium hydroxide* until a precipitate persists on stirring. Dissolve the precipitate in *hydrochloric acid* added dropwise, avoiding an excess over that necessary to secure a clear solution. Heat to 50°, then add with stirring an excess of *50% ammonium acetate solution* and 4 ml. of *glacial acetic acid.* Collect on an ashless paper the precipitate, which consists of aluminum phosphate mixed with a small amount of iron phosphate, and wash with hot water. Ignite at moderate redness in a platinum crucible and weigh.

Reduction and Titration. To the mixed phosphates contained in the crucible, add 10 parts of *sodium carbonate,* heat to fusion, cool, and dissolve in *dilute sulfuric acid.* Reduce with *hydrogen sulfide* or *zinc,* titrate with *standard 0.1 N potassium permanganate solution,* and calculate the iron as Fe_2O_3.

Phosphoric Acid Determination. In the same solution, after titration of the iron, determine phosphoric acid by the molybdic method.

Calculation. Subtract the sum of the weights of Fe_2O_3 and P_2O_5 from the weight of the mixed phosphates, thus obtaining the weight of Al_2O_3. Calculate the percentage of Al_2O_3 and Fe_2O_3.

Calcium

Determine the calcium as oxalate in the acid filtrate from the iron oxide and alumina without neutralizing (Official). See also Complete Ash Analysis (Part I, C8*a*).

Magnesium

To the filtrate from the calcium oxalate above, add *ammonium hydroxide* to slight alkaline reaction, then to one-sixth the final volume, stirring vigorously. Filter, ignite, and weigh as described under Complete Ash Analysis (Part I, C8*a*).

If magnesium is present, it is due to impurity in the acid phosphate or, less likely, some other constituent.

Sulfur (Sulfuric Acid), Potassium, and Sodium

Process. *Sulfuric Acid Precipitation.* Evaporate nearly to dryness a 100-ml. aliquot of the solution prepared as described

under Sand above, representing 1 g. of the sample, and dilute the slightly acid solution to about 100 ml. Heat to boiling, add *barium chloride solution* sufficient to precipitate the sulfuric acid, let stand overnight in a warm place, filter on paper, wash with hot water, ignite, and weigh.

Sulfuric acid, as shown by Winton, Ogden, and Langley,[37] may also be accurately determined in the direct acid solution, the dextrose formed by the hydrolysis of the starch being without influence (Official).

Calcium and Phosphoric Acid Precipitation. To the filtrate from the barium sulfate above, add *solid barium hydroxide* to strong alkaline reaction, thereby, in the absence of considerable sulfate, precipitating the lime as phosphate and the excess of phosphoric acid as barium salt. In powders containing a large amount of calcium sulfate as filler, the lime may be in excess of the phosphoric acid and the excess will remain in solution as chloride. Allow to settle, filter, and wash with hot water.

Barium Precipitation. Heat the filtrate to boiling and add sufficient *ammonium carbonate solution* to precipitate all the barium and, if present, calcium. Filter, wash with hot water, evaporate the filtrate on the water bath to apparent dryness, then heat for at least an additional 30 minutes to insure complete removal of water, thereby preventing decrepitation during heating over a naked flame below redness, which is next carried out until all ammonium salts are driven off.

Alkali Chloride Separation. Take up the residue in a little hot water, add a few drops of *ammonium carbonate solution*, and remove any precipitate or residue by filtering on a small paper into a tared platinum, quartz, or porcelain dish. Evaporate the filtrate, ignite below redness, cool in a desiccator, and weigh the mixed alkali chlorides (NaCl and KCl).

Potassium Determination. Determine potash (which is commonly present only in tartrate powders) as potassium platinichloride as described under Ash Analysis (Part I, C8*a*).

CALCULATION. Use the formulas:

$$BaSO_4 \times 0.3430 = SO_3$$

$$K_2PtCl_6 \times 0.1938 = K_2O$$

$$K_2PtCl_6 \times 0.3067 = NaCl$$

Deduct the weight of NaCl from that of NaCl + KCl to obtain the weight of NaCl; $NaCl \times 0.5303 = Na_2O$.

PHOSPHORUS

(Phosphoric Acid)

In a new aliquot of the filtrate from the sand above, determine phosphoric acid by the molybdic gravimetric method.

SODIUM HYDROGEN PYROPHOSPHATE

Standard Brands Zinc Sulfate Volumetric Method.[38] The method was developed at the Chicago Laboratory of the Royal Manufacturing Branch of Standard Brands Incorporated for the analysis of acid sodium pyrophosphate and pyrophosphate baking powders. It depends on the reaction

$$Na_2H_2P_2O_7 + 2ZnSO_4 \longrightarrow H_2SO_4 + Zn_2P_2O_7 + Na_2SO_4$$

PROCESS. *Direct Titration.* Weigh 1.111 g. of the sample into a 250-ml. beaker. Add 100 ml. of water, 7 drops of *0.04% bromophenol blue indicator* and 7.5 ml. of *0.2 N hydrochloric acid.* Add *0.2 N sodium hydroxide solution* to the proper blue color. Note the color as this will be taken as the end-point.

Back Titration. Add 5 g. of *zinc sulfate* or its equivalent as a solution. Stir well and titrate the released sulfuric acid with *0.2 N sodium hydroxide solution.* Use 1 drop of *bromophenol blue* on a spot plate as an external indicator. Record only the milliliters of

standard alkali used to bring back the color to its original blue.

Calculation. Calculate the percentage (P) of $Na_2H_2P_2O_7$ by the formula

$$P = \frac{0.0222 \times 100}{1.111} \times V = 2V$$

in which 0.0222 is milligrams of $Na_2H_2P_2O_7$ corresponding to 1 ml. of 0.2 N alkali and V is corrected milliliters of 0.2 N alkali used in the titration.

Arsenic

See Part I, C8*b*.

Lead

See also Part I, C8*b*.

Lead from the lining of storage and settling tanks was at one time an impurity in baking powder chemicals.

Victor Chemical Works Colorimetric Method. Reagents. *Alkaline Ammonium Acetate Solution.* Dissolve 390 g. of ammonium acetate in 1800 ml. of water and 150 ml. of NH_4OH.

Standard Lead Nitrate Solution. Dissolve 0.160 g. of $Pb(NO_3)_2$, dried over H_2SO_4, in 1 liter of water; 1 ml. = 0.0001 g. of lead.

Process. A. Acid Phosphate, Phosphate, and Alum-Phosphate Powders. *Lead Precipitation.* Boil 1 to 2 g. of the sample with 10 to 15 ml. of water and 2 to 3 ml. of *sulfuric acid,* continuing the boiling with replacement of water until the starch is hydrolyzed. After cooling, add 30 to 40 ml. of *ethanol,* stir, and allow to settle overnight. In case sodium aluminum sulfate separates out, add sufficient water to dissolve it.

Alkaline Acetate Digestion. Collect the lead sulfate on a filter and wash with 75 to 80 ml. of *ethanol* until acid-free. After drying, transfer the bulk of the precipitate to a crucible, digest with hot *alkaline ammonium acetate solution,* filter through the paper previously used, and wash with small portions of the hot *reagent.*

Color Formation. Cool the filtrate, make up to 50 ml., and add 1 ml. each of *10% potassium cyanide solution, 1% gelatin solution,* and *colorless ammonium sulfide solution.*

Color Reading. Compare the color with that of a suitable volume of *standard lead nitrate solution,* mixed with the same volume of *alkaline acetate, cyanide, gelatin,* and *ammonium sulfide* used in the actual analysis.

B. Tartrate Powders and Cream of Tartar. Shake 10 g. of the sample with 50 ml. of water and 40 ml. of *2 N ammonium hydroxide.* Dilute to 100 ml., mix, and filter through a dry paper. Mix 50 ml. of the filtrate directly, without precipitation as sulfate, with 1 ml. each of the four *reagents* named above and compare with a *solution of the standard* treated in like manner, but containing about the same amount of lead-free cream of tartar as the unknown.

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L. SALT

The analysis of salt involves determination of the constituents other than sodium chloride from which by difference the content of the pure substance may be calculated. Sulfates and chlorides of calcium and magnesium are the common impurities present in the brands of the commercial product designed for table use in such small quantities as to be best determined in a rather large charge—say 5 g.—or an equivalent aliquot. A volumetric determination of chlorine in the sample and qualitative tests for impurities often suffice. The methods given in Part I, C8*a*, are adequate. Rock salt, designed for cattle feeding, may contain impurities in such amount as to warrant an exhaustive analysis.

Iodized salt, designed to supplement the deficiency in the water of goiter-prevalent regions, is supplied for both human and animal consumption.

Both Gronover and Wohnlich [1] and Andrew and Mandeno [2] describe essentially the same modification of the von Fellenberg process.

PROCESS. *Solution and Oxidation.* Dissolve 100 g. of the sample in 500 ml. of water and filter from any insoluble matter. To 100 ml. of the filtrate, add 1 ml. of *1.0 N hydrochloric acid* and 1 ml. of *bromine water* to oxidize the iodine to iodate. Boil vigorously for 10 minutes with the addition of a few pieces of *pumice stone* to remove the excess of bromine, replacing from time to time the water, and cool to 10°.

Titration. Add 1.5 ml. of *85% phosphoric acid,* 0.2 g. of *potassium iodide,* allow to stand in the dark 3 minutes, and titrate with *0.004 N standard sodium thiosulfate solution.*

CALCULATION. Calculate as described in Part I, C8*b*.

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